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NEW MICROBIAL INDICATORS OF WASTEWATER CHLORINATION EFFICIENCY

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16

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ABSTRACT

The coliform group of indicator organisms has a relatively low resistance to chlorine when compared to pathogens such as enteric viruses and protozoan cysts. Consequently, an effort has been initiated to find a new chlorine resistant bioindicator of wastewater chlorination efficiency. Organisms surviving chlorination of wastewater effluents to a free chlorine residual were collected for study by plating on various growth media. This procedure provided 135 chlorine resistant isolates for further examination. A yeast and two different acid-fast bacilli were found to be resistant to chlorination in the range considered necessary for the inactivation of pathogens, including viruses. One acid-fast bacillus survived 2.0 mg/ℓ free chlorine for 67 min while the other survived 1.0 mg/ℓ free chlorine for 15 min. The yeast resisted 1.0 mg/ℓ free chlorine for 20 min. In comparison, a pure culture of *E. coli* failed to survive 5 min contact with 0.03 mg/ℓ free chlorine. Yeasts were found to be easily cultured on acidified yeast extract-malt extract agar. Acid-fast staining of colonies on membrane filters incubated on mineral-propionate medium, following sample pretreatment with oxalic acid and NaOH provided a selective assay technique for acid-fast organisms. Significant quantities of yeasts and acid-fast organisms were found in wastewater effluents and yeasts have been isolated from stools. The chlorine resistance and other characteristics of the yeasts and acid-fast bacilli under study suggest that they show promise as bioindicators for wastewater chlorination efficiency for the more chlorine resistant pathogens. This report was submitted by the University of Illinois at Urbana-Champaign, Department of Civil Engineering, in fulfillment of Project Number 17060EYZ under the sponsorship of the Environmental Protection Agency.

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CONTENTS

Section		Page
I	Conclusions	1
II	Recommendations	3
III	Introduction	5
	Relative Resistance of Coliforms and Viruses to Chlorine	5
	Relative Resistance of Coliforms and Vegetative Bacterial Pathogens to Chlorine	6
	Resistance of Cysts to Chlorine	6
	Resistance of Acid-Fast Bacilli to Chlorine	7
	Prevalence of Yeasts and Acid-Fast Organisms in Fecal Material and Wastewater	8
	Objectives of this Study	10
IV	Materials and Methods	13
	Measurement of Chlorine Residuals	13
	Isolation of Chlorine Resistant Organisms	13
	Culturing of Chlorine Resistant Isolates	14
	Screening of Isolates for Chlorine Resistance	14
	Detailed Chlorination Studies	16
	Enumeration of Potential Indicator Organisms in Human Fecal Material and Wastewater	16
V	Results and Discussion	23
	Isolates Examined for Chlorine Resistance	23
	Further Examination of Isolates from Chlorinated Wastewater	23
	Enumeration of Yeasts in Fecal Material and Wastewater	37

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CONTENTS (continued)

Section		Page
	Selective Techniques for Enumeration of Acid-Fast Bacilli	50
VI	Acknowledgements	59
VII	References	61
VIII	Publications and Patents	67

FIGURES

		Page
1	Response of Isolate No. 30 to Free Chlorine Residuals Ranging from 0.1 to 1.0 mg/ℓ	27
2	Response of Isolate No. 30A to Free Chlorine Residuals Ranging from 0.1 to 1.0 mg/ℓ	28
3	Response of Isolate No. 82 to Free Chlorine Residuals Ranging from 0.1 to 1.0 mg/ℓ	29
4	Response of Isolate No. 132 to Free Chlorine Residuals Ranging from 0.1 to 1.0 mg/ℓ	30
5	Response of Isolate No. 134 to Free Chlorine Residuals Ranging from 0.5 to 2.0 mg/ℓ	32
6	Free Chlorine Residuals and Contact Times Necessary for 99.9 Percent Kill for Isolates No. 134, No. 30A, No. 132, and No. 30 as well as for Organisms Compiled by Berg	33
7	Comparison of Response of *E. coli* ATCC-11229 to Free Chlorine with Data from Butterfield *et al.*	35
8	Chlorination Study of Isolate No. 30 Seeded in Diluted Secondary Effluent	38

TABLES

No.		Page
1	Conditions of Isolation and Properties of Representative Isolates	24
2	Chlorination Study of Isolate No. 30 Seeded in Secondary Effluent	36
3	Examination of Fecal Samples for Total Plate Count and Yeasts	39
4	Statistical Analysis of Fecal Yeast Densities	40
5	Yeast Densities in Raw Municipal Wastewater	42
6	Yeast Densities in Activated Sludge Effluent	43
7	Yeast Densities in Trickling Filter Effluent	44
8	Total Bacteria and Coliform Content in Raw Municipal Wastewater	45
9	Total Bacteria and Coliform Content in Activated Sludge Effluent	46
10	Total Bacteria and Coliform Content in Trickling Filter Effluent	47
11	Pre-Screening for Chlorine Resistance of Yeasts Isolated from Unchlorinated Wastewater Samples or Stools	49
12	Effect of Selective Pretreatment Methods on Growth of Acid-Fast Isolates	52
13	Effect of Selective Media on Growth of Acid-Fast Isolates	54
14	Effect of Sulfa Drugs in Selective Media on Growth of Acid-Fast Isolates	56

SECTION I

CONCLUSIONS

1. *Escherichia coli* is not sufficiently resistant to chlorine to be a useful bioindicator of wastewater chlorination efficiency for chlorine resistant pathogens such as viruses.

2. Contact of wastewater for 5 min with sufficient chlorine to satisfy a portion or all of the chlorine demand is a feasible means of screening out chlorine sensitive organisms from wastewater. Moderately to highly chlorine resistant organisms surviving this procedure may then be collected for detailed study.

3. The degree of chlorine resistance of organisms collected in the screening procedure is not well correlated with the chlorine dosage used.

4. The majority of chlorine resistant organisms collected as potential new indicator organisms were gram positive sporeformers; these were not studied further due to the dependence of the sporeforming property on generally uncontrollable biological factors.

5. A yeast, Isolate No. 30, and two acid-fast bacilli, Isolate Nos. 132 and 134, were found to be sufficiently resistant to free chlorine residuals in chlorine demand free water to be potentially useful as bioindicators of wastewater chlorination efficiency.

6. Isolate Nos. 30, 132 and 134 show resistance to free chlorine equal to or greater than that reported for resistant pathogens such as Coxsackie A2 virus; the isolates are able to withstand 1.0 mg/ℓ free chlorine in chlorine demand free water for up to 15, 15, and 60 min contact time, respectively.

7. Yeast organisms could be selectively cultured from secondary effluents on yeast extract-malt extract glucose agar adjusted to pH 3.5 and incubated at 20°C; however, growth of acidophilic molds caused some interference in enumeration.

8. Yeasts were isolated from human fecal material in concentrations up to 10^4 yeasts per g in 80 percent of 31 samples tested.

9. Yeasts were isolated from wastewater in average concentrations of 1500, 220, and 420 yeast per mℓ of raw wastewater, activated sludge and trickling filter effluents, respectively.

10. Use of sample pretreatment by acidification with 2.5 percent oxalic acid for 5 to 10 min followed by neutralization with 2 percent NaOH considerably reduced the number of contaminating organisms for the enumeration of acid-fast bacteria in wastewater.

11. A method for acid-fast staining of membrane filters was developed and, when combined with oxalic acid-NaOH pretreatment and selective media, provided a sensitive and selective method for enumeration of acid-fast bacteria in wastewater.

12. Limited studies of raw wastewater indicated the presence of acid-fast bacteria in concentrations of 137 to 500 per mℓ.

SECTION II

RECOMMENDATIONS

1. Chlorine resistant organisms isolated thus far require further evaluation as to their resistance in a variety of wastewater effluent types.

2. The membrane filter technique for enumerating acid-fast bacilli should be thoroughly studied as to its sensitivity, reliability and accuracy in highly contaminated samples such as wastewater.

3. A method for inhibiting mold growth in assaying wastewater effluents for chlorine resistant yeasts is needed.

4. The quantities of proposed indicators in fecal material, and various types of wastewater effluents, including AWT type effluents, in a variety of climatic and geographical situations, should be determined.

5. Taxonomic studies of the proposed indicators should be carried out.

6. Proposed indicators should be evaluated under field conditions in a variety of water quality situations for appropriate sampling methods, the need for and type of sample concentration techniques, and proper sample storage procedure.

7. Laboratory scale studies of the response of the proposed indicator organisms to AWT processes is necessary to provide information on their usefulness in water reuse schemes.

8. The response of the proposed indicators to disinfectants other than chlorine should be studied.

9. The resistance of the proposed indicator to disinfectants should be directly compared to that of resistant pathogens; comparison of the relative survival rates of the indicators and pathogens in receiving water is needed.

SECTION III

INTRODUCTION

The presence of pathogenic organisms in the effluents of conventional secondary and advanced wastewater treatment operations which may be resistant to the widely used chlorine disinfection process is a matter of increased concern. It is presently anticipated that direct reuse of reclaimed wastewater effluents for potable water supplies will be necessary in the foreseeable future in many areas of this country due to rising population and increased per capita water demand. A reliable biological indicator of the efficiency of the wastewater disinfection step is one approach that can be used to protect the health of populations using reclaimed wastewater for potable water supplies and also to alleviate public health fears of people using waters receiving chlorinated waste effluents for other purposes such as contact recreation.

RELATIVE RESISTANCE OF COLIFORMS AND VIRUSES TO CHLORINE

The literature indicates that the presently used coliform group of organisms is far too sensitive to chlorine to be of much value in indicating the potential presence of chlorine resistant infective agents such as viruses. At low temperatures, i.e. 0° to 6°C, polio 1 and Coxsackie A2 viruses are much more resistant to chlorine than *Escherichia coli* while adenovirus 3 is apparently more sensitive (1, 2). Nupen (3) concluded that coliform findings should not serve as a measure of the virus contamination in water and sewage. Liu *et al.* (4) studied the chlorine resistance of 20 human enteric viruses seeded in Potomac River water. The least chlorine resistant virus, reovirus I, required only 2.7 min contact with 0.5 mg/ℓ free chlorine for a 99.99 percent kill, assuming first order kinetics. The most chlorine resistant viruses were poliovirus 2 and Coxsackie B5 which required 40 min to reach the same kill as the reovirus under the same conditions and assumptions. Experimentally, Coxsackie A5 and echovirus 12 survived these conditions for 53.5 and more than 60 min, respectively. Kruse´ (5) compared the chlorine resistance of bacterial virus f2 and *E. coli* under a variety of experimental conditions. *E. coli* was found to be considerably more sensitive to inorganic and organic chloramines at 0° to 4°C than the model virus. For example, the density of *E. coli* decreased nearly 5 logs in less than 1 min while the model virus f2 was inactivated to slightly more than 1 log following 10 min contact with 10 mg/ℓ chlorine in the presence of 10^{-3} M NH_4Cl. The work of Kruse´ demonstrated that inactivation rates are considerably reduced for both *E. coli* and virus when the combined chlorine residual is organic chloramine as opposed to inorganic forms. Kjellander and Lund (6) have reported similar findings for *E. coli* and poliovirus 3. Burns and Sproul (7) found that bacteriophage T2 was considerably more resistant to free chlorine than

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E. coli. The resistance of viruses to chlorine appears to vary widely. Thus, if a full assessment of the relative value of an indicator organism is to be made, the chlorine resistance of all enteric viruses must be studied. The data that do exist suggest that, with few exceptions, viruses are more resistant to chlorine than coliforms by a considerable margin.

RELATIVE RESISTANCE OF COLIFORMS AND VEGETATIVE BACTERIAL PATHOGENS TO CHLORINE

The resistance of wastewater pathogens other than virus to chlorine is quite variable. Kabler (8) compared the chlorine resistance of *E. coli*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Salmonella typhosa*, and *Shigella dysenteriae* and *sonnei*. At pH 7 and 20° to 25°C, resistance to free chlorine was approximately the same for *E. coli* and *P. aeruginosa* but was less than that found for *S. typhosa* and *Shigella dysenteriae*. Resistance to combined chlorine was comparable for *E. coli*, *P. aeruginosa*, *Shigella sonnei*, and *S. typhosa*, while *Shigella dysenteriae* was more sensitive than the others. *A. aerogenes* was more resistant to both combined chlorine and free chlorine than the other bacteria studied. Tonney *et al.* (9) studied strains of *Pneumococcus*, *Streptococcus*, *Salmonella*, and *Shigella* to determine the dosage of chlorine required to kill vegetative cells within 15 to 30 sec. Only strains of *E. coli* and *Strep. hemolyticus* could withstand a residual of 0.25 mg/ℓ. Ritter and Treece (10) compared the chlorine resistance of 12 enterococcal strains to coliforms and found the majority able to withstand 0.1 to 0.2 mg/ℓ free chlorine for 1 hr and a 10 min exposure to 0.8 to 1.0 mg/ℓ. They observed that strains of *Staphylococcus* and *Streptococcus* were 5 to 20 times more resistant to chlorine than coliforms. Favero and his associates (11, 12) showed *Staph. aureus* to be more resistant to chlorine than either coliforms or *P. aeruginosa*. However, for the majority of vegetative bacteria which have been examined, it would appear that their resistance to chlorine and chloramine is essentially the same or less than that of the coliform bacteria.

Spores are generally considerably more resistant to chlorine than vegetative cells. Phillips (13) reported spores to be 10,000 times more resistant to hypochlorites than vegetative cells. Similar findings were noted by Brazis *et al.* (14). Tonney *et al.* (15) observed that a 280 mg/ℓ dosage of chlorine was necessary to obtain a 15 to 30 sec kill time for *Bacillus vulgatus* spores.

RESISTANCE OF CYSTS TO CHLORINE

Cysts of protozoans, like spores, are a resting stage of the parent organism and have a reproductive function as well. Being similar to spores, cysts are generally quite resistant to chlorine. *Entamoeba*

histolytica and species of the *Hartmanella-Naegleria* group are pathogenic protozoans of man capable of being transmitted by the water route (16). Chang (17) reported that cysts were 160 times more resistant to HOCl than *E. coli* and 9 times more resistant than the hardier enteroviruses. Hypochlorous acid residuals at 1.2 mg/ℓ for 30 min contact were required to kill 100 percent of *E. histolytica* cysts at 23°C in one study (18). Stringer (16) compared the cysticidal properties of 2 mg/ℓ each of free, combined inorganic and combined organic chlorine at pH 7 and 27°C and observed that a 10 min and 40 min contact time was necessary for a 99 percent kill for the first two categories, while organic chloramines failed to destroy more than 50 percent of the cysts even after 100 min contact. Cysticidal activity was greatest at low pH. Although the high degree of resistance which amoebic cysts have to chlorine should be a matter of concern, it should be noted that they are generally effectively removed by coagulation-sedimentation and filtration (19). In addition, they occur in cyst contaminated waters at levels not likely to be greater than 5 cyst per mℓ (20). Since the cysticidal chlorine dose greatly depends on the quantity of cysts present, and, since most investigations have employed cyst concentrations greatly in excess of those that might be expected to occur naturally, it is likely that reported cysticidal doses are greater than those required in the field.

RESISTANCE OF ACID-FAST BACILLI TO CHLORINE

Mycobacterium spp. and *Nocardia* spp. are acid-fast organisms which have been recovered from fecal material and wastewater (21, 22). *Mycobacterium tuberculosis* is of major concern from a public health standpoint while other members of the acid-fast genera may be pathogenic depending on various biological factors. Mycobacteria differ from other vegetative bacteria in that they have protective layers of mycolic acid and lipids in their cell walls (23). These cell wall constituents confer a degree of resistance of mycobacteria to chemical agents such as chlorine that are generally not found in other vegetative bacterial groups. Musehold (24) found 17 mg/ℓ was insufficient while 340 mg/ℓ was sufficient to kill tubercle bacilli following a 2 hr contact in sewage samples. Kabler (25) found that up to 2 hr contact with 1 to 2 mg/ℓ chlorine was necessary to achieve a 99 percent inactivation of *M. tuberculosis*. Greenberg and Kupka (26) reviewed about 15 different studies which led them to conclude that a chlorine dosage of 20 mg/ℓ with a 2 to 3 hr contact time or a chlorine residual of at least 1 mg/ℓ after 1 hr contact were the conditions necessary to destroy all tuberculosis organisms. If these organisms are of concern from a public health standpoint, then higher chlorine residuals and longer contact periods are required to inactivate tuberculosis organisms in comparison to those needed to destroy enteric bacteria.

PREVALENCE OF YEASTS AND ACID-FAST ORGANISMS IN FECAL MATERIAL AND WASTEWATER

Two groups of organisms which this study has shown to have potential as chlorine resistant bioindicators are yeasts and acid-fast bacilli. Studies of their occurrence in fecal material and wastewater are rather limited, and knowledge of acid-fast organisms in these materials has focused principally on the tubercle bacilli.

Cooke (27) reviewed the occurrence of yeasts in a variety of habitats and reported that they occur commonly in the alimentary tracts of higher animals which ingest them with their food. Saprophytic yeasts are able to pass through the digestive tract relatively unscathed by the acid conditions in the stomach. Asporogenous type yeasts appear to predominate in human feces. While fecal yeasts are generally retained no longer than ingested food, pathogenic strains of *Candida albicans* can maintain residence and multiply in the intestine. The majority of yeasts, however, are probably transitory inhabitants of the intestine, not multiplying to any great extent (28). Benham and Hopkins (29) isolated species of *Candida*, *Cryptococcus*, *Geotrichium*, *Saccharomyces*, and *Zygosaccharomyces* from feces of normal persons with *Candida albicans* isolated from 18 percent and *Geotrichium* from 41 percent of the cases studied.

Cooke and his associates (27,30,31,32) have studied the occurrence of yeasts in various wastewater treatment processes. The yeast population was found to increase somewhat as wastewater passed through a trickling filter treatment plant with yeasts maintaining growths on the stones of the trickling filter. Yeasts were found to be viable in wastewater sludges and to survive anaerobic sludge digestion at 98°F, as well as drying on sludge drying beds. Cooke and Pipes (31) examined 18 waste treatment plants treating both domestic and industrial wastewaters. Of 20 species of fungi isolated, only 1.18 percent of the colonies represented yeast species (*Candida* spp., *Trichosporon* spp., *Rhodotorula* spp.). It should be noted that the techniques employed were designed for isolation of filamentous fungi and would not necessarily be suitable for culturing of yeasts. Cooke and Matsuura (32) qualitatively studied yeasts and fungi in waste stabilization ponds and reported isolation of 212 species of fungi of which about 50 were yeasts. Yeasts multiplied rapidly in the ponds and were abundantly present in the waters, sludges, and soils of the pond system.

It may be concluded that one may expect yeasts to be present in fecal material and in wastewaters, including waste treatment system effluents. Little quantitative information is available to assess the total potential population of yeasts in such materials. Qualitative reports suggest a wide variety of yeast species may be present with members of the genera *Rhodotorula*, *Torula*, *Torulopsis*, *Candida*, and *Trichosporon* frequently encountered.

Acid-fast organisms are of interest because of their reported chlorine resistance and the possible pathogenicity of some *Nocardia* and *Mycobacterium* species. Reports of tubercle bacilli in feces date back to at least 1900. Reports of acid-fast bacilli in feces other than the tubercle bacilli are quite sparse. Klose (33) found acid-fast bacilli in the stools of 55 of 60 patients showing sputum positive for tubercle bacilli. No acid-fast bacilli were found in 12 fecal specimens from persons with sputum negative for tubercle bacilli. Alexander (34) found acid-fast organisms in stools of 38 of 45 pulmonary tuberculosis patients. Laird *et al.* (35) reviewed similar studies and reported that the majority of patients showing positive sputum specimens also provided stool specimens which contained acid-fast organisms. Acid-fast organisms were not frequently found in the feces of healthy persons. Wilson and Rosenberger (36) detected acid-fast organisms in 21 percent of over 1000 fecal samples from apparently healthy individuals. Laird *et al.* (34), on the other hand, found acid-fast organisms in the feces of only 2 of 54 persons with negative sputum. It should be noted that these findings are quite outdated in terms of techniques employed. It is quite possible that the use of more modern methods not specifically aimed at finding tubercle bacilli but more generally towards isolating all potential acid-fast organisms would show a greater percentage of the healthy population to be excreters of acid-fast organisms.

Several investigations on the presence of tubercle bacilli in wastewater have appeared in the literature. Musehold (24) added tubercle bacilli to river water, sewage, and sewage sludge and exposed the samples to prevailing environmental conditions. Viable bacilli were recovered from river water and sewage sludge even after 5 months and 6 1/2 months exposure to these conditions, respectively. Bacilli were also demonstrated in the effluent from a secondary wastewater treatment plant. Jensen and Jensen (21) were consistently able to recover viable *M. tuberculosis* in effluents from sanatoria following biological treatment, even when the populations of aerobic enteric bacteria had been reduced to 0.2 percent of their original numbers. These authors also determined that 10 of 10 samples each from raw sewage and activated sludge effluent from a sanitorium treatment plant were positive for tubercle bacilli while only 1 of 10 samples from the chlorinated effluent contained detectable quantities of these organisms. Dosage was 10 mg/ℓ chlorine with a contact in excess of 3 hr. Pramer *et al.* (22) reported that avian tubercle bacilli seeded into raw wastewater was not removed by 6 hr of settling. Chemical coagulation with ferric chloride at pH 5.0 resulted in removal of 99 percent of the bacilli as did sand filtration. On the other hand, aeration of the wastewater for 24 hr in laboratory biological treatment units had little effect on the density of tubercle bacilli. Free chlorine residual disinfection of the wastewater was necessary to achieve significant reduction of the bacilli. The authors noted that free residuals and dosages used were considerably higher than that normally maintained for disinfection of ordinary municipal wastewater.

The authors studied a tuberculosis sanitorium trickling filter plant and found 1500 *M. tuberculosis* organisms per mℓ in the raw waste which were reduced to 10 per mℓ in the chlorinated plant effluent. However, the raw sludge had 100,000 organisms per mℓ indicating that while removal may have appeared to be efficient, tremendous quantities of viable pathogens had been concentrated in the sludge. Digestion of the sludge was only able to reduce the *M. tuberculosis* density to 10,000 per mℓ. Müller (37) reported that raw wastewater contained 5 to 100 tubercle bacteria per liter compared to 100 times as many *Salmonella* organisms in the same volume. She stated that tubercle bacteria must be considered to be a regular component of urban wastewater, even without known sanitaria sources. The investigator found 90 percent of the raw wastewater and 50 percent of the settled wastewater samples to be positive for tubercle bacilli. On the other hand, sludges subjected to 30°C under septic conditions showed no tubercle bacilli. Sludge samples taken from drying beds used for waste activated sludge were 44 percent positive even after 30 days drying time. It would appear from these studies that while biological treatment may be counted on for some removal of acid-fast organisms, both the effluent and the resulting sludges will contain viable organisms. Chlorination of the effluent with high dosage at extended contact periods may provide a safe effluent.

OBJECTIVES OF THIS STUDY

The major objective of chlorination of wastewater effluents is to produce an effluent that is acceptable from a public health standpoint. In order to assess the efficiency of wastewater chlorination in terms of destruction of pathogens, there is a need for a reliable bioindicator which is at least as resistant to chlorine as the most chlorine resistant pathogens. The bioindicator should also be rapidly and unambiguously quantifiable in chlorinated effluents by simple and easily applied techniques. Since it would appear that coliforms, the most commonly applied group of bioindicators, do not meet the criterion of being as resistant to chlorine as the most resistant pathogens, the suitability of coliforms for judging the efficiency of wastewater chlorination may be seriously questioned. This is particularly true where wastewater reuse schemes are being considered, since, in these situations, protection of the public health is paramount.

The coliform organisms have been quite helpful in the past in providing information on the potential presence of bacterial pathogens in many types of waters. However, it would seem appropriate in light of their inability to satisfy the public health needs in many situations to re-evaluate their application on a case-by-case basis. It is suggested, therefore, that the indicator organisms should be selected for use on the basis of the purpose and information required, e.g., the inactivation of resistant pathogens by chlorination. It was with this purpose in mind that this study was undertaken.

It need not be emphasized that the majority of results obtained in this study are of a negative nature. This is to be expected in a search study of this type. It may be pointed out that several decades were required to develop the coliform test to its present state of refinement, with many false leads and pitfalls along the way. The negative results presented here may help other investigators to avoid the expenditure of research efforts in areas that show little promise of a successful outcome. On the other hand, it is felt that several promising developments towards the realization of a new indicator of wastewater chlorination efficiency have arisen from this study. The original objective of the study to find an organism(s) that is as resistant to chlorine as the most resistant pathogens and to develop rapid, simple, sensitive and selective procedures for their enumeration in secondary and advanced waste treatment effluents has been met at least in part. Further study will be required to refine techniques and to test the validity of the proposed wastewater indicators under a variety of field conditions. The techniques developed in this study have provided several candidate indicator organisms for measurement of chlorination efficiency and, thus, have considerably expanded the available alternatives in assessing the public health quality of chlorinated effluents.

SECTION IV

MATERIALS AND METHODS

MEASUREMENT OF CHLORINE RESIDUALS

The measurement of chlorine residuals in secondary effluents was found to be most satisfactory when the DPD (N,N-dimethyl-p-phenylene-diamine monohydrochloride obtained from Eastman Kodak Company, Rochester, New York) colorimetric method (38,39,40,41) was used because both free and total chlorine residuals can be determined in the same samples within a 2 min period. It should be noted that high levels of chloramines may interfere with this procedure, yielding a higher apparent free chlorine residual than is actually present. Oxidation-reduction potential measurements were employed to confirm the reliability of this method.

It is generally accepted that the amperometric method of measuring chlorine residuals is the preferred procedure in studying the response of microorganisms to chlorine when precise information is desired (7,39,42). Burns and Sproul (7) conducted a study in which chlorine residuals determined by amperometric titration showed a constant relationship to organism destruction whereas orthotolidine-arsenite (OTA) residuals did not. Chambers (43) found that if the amperometer was "sensitized" before use by repeated chlorine residual measurements, i.e. 15 times, using a solution having approximately the same concentration of chlorine as the test solution, chlorine concentrations as low as 0.005 mg/ℓ could be detected. An amperometric titrater (Wallace and Tiernan, Inc., Belleville, New Jersey) was used to measure chlorine residuals in the controlled chlorination studies carried out with chlorine demand free water. The procedure for amperometric titrations described in *Standard Methods* (44) was followed.

ISOLATION OF CHLORINE RESISTANT ORGANISMS

Several activated sludge and trickling filter effluent samples from the East Side Treatment Plant of the Urbana and Champaign Sanitary District and a sample of oxidation pond effluent from the Decatur Sanitary District Sewage Treatment Plant were the main sources of potential indicator organisms. These samples were filtered through several layers of cheesecloth and stored at 10°C for never more than 5 hr before chlorination. The different effluent samples were chlorinated either to satisfy part of the demand by adding from 6 to 63 mg/ℓ of chlorine or to reach a free chlorine residual of 0.15 to 8.0 mg/ℓ by adding from 75 to 176 mg/ℓ of chlorine as sodium hypochlorite for contact periods of 5 to 30 min at either room temperature or 20.5°C. Following dechlorination with an excess of 0.05N sodium thiosulfate, aliquots were plated on several types of media. These included nutrient agar and tryptone-glucose agar

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from Difco Laboratories (Detroit, Michigan) and trypticase-soy agar, WL Differential agar, and Middlebrook and Cohn 7H10 agar from BBL (Cockneysville, Maryland). Media prepared in the laboratory included starch agar (0.5 percent peptone, 0.5 percent yeast extract, 0.3 percent soluble starch, 1.5 percent agar), nitrate agar (1 percent peptone, 0.5 percent yeast extract, 0.4 percent potassium nitrate, 1.5 percent agar), and succinate agar (0.2 percent NH_4NO_3, 0.1 percent K_2HPO_4, 0.02 percent $MgSO_4$, 0.5 percent sodium succinate, 1.5 percent agar). The agar was obtained from Difco Laboratories and the other chemicals from Mallinckrodt Chemical Works (St. Louis, Missouri). Double strength nutrient agar adjusted separately to pH 3, 5, 7, and 9 was also used. The streaked plates were incubated at 10°, 20°, 37°, and 45°C for 24 to 96 hr.

CULTURING OF CHLORINE RESISTANT ISOLATES

Organisms that grew on the originally inoculated plates, following chlorination of the effluent, were examined by a phase-contrast microscope for morphological characteristics, Gram stained, and subcultured to obtain pure cultures. Stock cultures were made on nutrient agar slants and stored at 5°C. Subcultures collected for detailed study were periodically transferred to fresh agar slants.

In preparation for controlled chlorination experiments, the extinction coefficient of each isolate was determined. This was accomplished by growing each organism in nutrient broth on a shaker and, after an appropriate incubation period, the culture was centrifuged and the cells washed twice and resuspended in phosphate buffered water at pH 7. The optical density of the suspension was measured at 660 n.m. in a Spectronic 20 spectrophotometer and various dilutions were plated in nutrient agar pour plates. The data obtained from pour plate colony enumeration were correlated with the optical density to obtain the extinction coefficient.

SCREENING OF ISOLATES FOR CHLORINE RESISTANCE

Pure cultures of isolates were examined as to their specific resistance to chlorine. Several types of water were considered for use as the basic suspending menstrum, including 1) raw ground water aerated to precipitate ferric iron, membrane filtered, and buffered to pH 7; 2) water prepared in the same manner as No. 1 but autoclaved after pH adjustment; 3) autoclaved distilled water buffered to pH 7; and 4) physiological saline prepared from deionized water buffered to pH 7. However, for various reasons these waters were found to be unsatisfactory. On the other hand, it was found that the requirements for these studies could be met best by using the chlorine demand free water as developed by Butterfield *et al.* (45), the complete preparation of which was described by Megregian (46).

The glassware was washed with Haemosol (Meinecke and Company, New York)

and then soaked in a strong chlorine solution (75-150 mg/ℓ) for at least 2 hr before rinsing at least 10 times with deionized water and finally with chlorine demand free water (43). Glassware used with chemicals, potentially toxic to the organisms under investigation, was soaked overnight in cleaning acid (35 mℓ of saturated sodium dichromate in 1 ℓ of concentrated sulfuric acid) and then rinsed with water. Sterilization was accomplished in a dry hot air oven at 190°C for 2 hr.

Prior to a chlorination experiment, the isolate to be examined was grown overnight in an aerated nutrient broth culture. The washed suspension was then diluted on the basis of cell concentration as determined by optical density measurements and 1 mℓ aliquots were added to the test flasks so as to have a final cell concentration of 10^3 to 10^4/mℓ in the flasks.

The response of the various isolates to chlorine was determined using essentially the same procedure described by Butterfield *et al.* (45). A determined amount of chlorine and a test organism were added to a number of sterile 1 ℓ erlenmeyer flasks, each containing 500 to 700 mℓ of sterile chlorine demand free water. Two control flasks were treated in the same manner except that one received only chlorine and the other only the test organisms. The flasks were incubated in a water bath at 20.5°C. The preliminary screening was originally performed using chlorine dosages of 0.1, 0.5, and 1.0 mg/ℓ, but was later changed to a single dosage of 0.5 mg/ℓ so that three or more isolates could be screened at one time. Later to facilitate the screening of isolates, chlorination experiments were done with a mixture of morphologically similar organisms in one reaction flask. Those isolates showing some resistance at 0.5 mg/ℓ free chlorine residual were then further screened as to chlorine resistance with the three chlorine concentrations mentioned above. The free chlorine residuals were measured by amperometric titration after 5 and 30 min contact. In this phase of study, 1 mℓ aliquots were removed from the test flasks at 5, 15, and 30 min intervals, dechlorinated with excess 0.025 N thiosulfate in a fivefold dilution, diluted tenfold further in pH 7 phosphate buffer, and plated on agar pour plates using an appropriate nutrient medium. Replicate plates of each dilution were made and incubated for 24 to 48 hr. Promising isolates were subjected to more detailed studies with chlorine dosages of 0.1, 0.3, 0.5, 0.7, and 1.0 mg/ℓ with removal of 1 mℓ aliquots at 5, 10, 15, 20, and 30 min intervals. Further studies employed higher chlorine concentrations and longer exposure times depending on the chlorine resistance of the particular isolate. The entire selection procedure from the chlorination of effluent to the final chlorination experiments using high chlorine dosages in chlorine demand free water, provided a means of continually eliminating organisms which were unsuitable due to low chlorine resistance and other undesirable properties.

DETAILED CHLORINATION STUDIES

Using the most promising organisms, six separate sets of experiments were carried out to obtain further information concerning their properties. The purpose of one group of experiments was to determine the length of time and concentration required for 99.9 percent kill of the test organism. The contact times were increased to as much as 200 min and the chlorine concentrations up to 2 mg/ℓ.

A culture of *E. coli* ATCC-11229, obtained from the National Environmental Research Center in Cincinnati, Ohio, was examined for its resistance using concentrations of free residual chlorine of 0.01 to 0.04 mg/ℓ at a cell concentration of 10^4/mℓ. Samples were taken following 1, 5, 10, 15, 20, and 30 min of exposure to chlorine. This organism was employed due to its previous use by other investigators so as to allow comparison of results.

Mixed culture chlorination experiments were carried out using yeast isolate No. 30 and *E. coli* ATCC-11229 to study survival at chlorine concentrations of 0.02, 0.1, and 0.5 mg/ℓ. These two organisms were added together in the same reaction flasks; the concentration of *E. coli* was 5000/mℓ and that of No. 30 was 1000/mℓ. Organism survival was determined after 1, 5, 10, 15, 20, and 30 min contact with the chlorine. To distinguish between the two organisms, samples were plated on EMB agar on which only *E. coli* gives the lactose reaction and on nutrient agar adjusted to pH 3.5 with d-tartaric acid (J. T. Baker Chemical Company, New Jersey) for detection of Isolate No. 30.

The appropriateness of a potential new indicator organism as applied to wastewater chlorination was evaluated through studies of its chlorine resistance when seeded in secondary effluent. In experiments involving the use of yeast Isolate No. 30, chlorine residual measurements were made using the DPD method. The apparent free chlorine residuals measured in these experiments may have been due, in part, to interference caused by high concentrations of chloramines. However, the alternative amperometric method was found to produce unreliable results due to an indistinct endpoint when used for chlorine measurements with the wastewater samples. In order to reduce the chlorine demand and the production of chloramines, wastewater effluents were diluted tenfold prior to chlorination. This dilution probably provided a quality water that might be similar to an AWT type effluent. Chlorine dosage could thus be markedly reduced while still allowing the attainment of free chlorine residuals. Thus, meaningful survival curves for resistance to free chlorine could be obtained.

ENUMERATION OF POTENTIAL INDICATOR ORGANISMS IN HUMAN FECAL MATERIAL AND WASTEWATER

For a test organism to be a suitable indicator of fecal contamination, it

should be routinely present in human fecal material and also in domestic wastewater. Therefore, both stool samples and wastewater were examined for the presence of yeasts and acid-fast bacteria, two groups of isolates which showed chlorine resistance at levels considered necessary for inactivation of pathogens, including viruses.

Yeast Enumeration in Fecal Material and Wastewater

Because the growth of the majority of bacteria and actinomycetes is inhibited at pH values less than 4.5 while yeasts and molds are not, a selective enrichment medium yeast extract-malt extract glucose agar (YMA) (yeast extract 0.3 percent, malt extract 0.3 percent, glucose 1 percent, peptone 0.5 percent, agar 2 percent) which had been adjusted to pH 3.5-4.0 with 1 N HCl was used as the medium of choice for yeast enumeration in both fecal samples and wastewater. Initially the acid medium used was potato dextrose agar adjusted to pH 3.5 with 10 percent tartaric acid. Wickerham (47) recommended that yeast extract-malt extract glucose agar (YMA) be used as the medium of choice for yeast enrichment when the pH is lowered to the acid range (pH 3.5-4.0). This medium was evaluated using yeast Isolate Nos. 30 and 30A. After daily examination for the growth of yeasts in three acid media, i.e. nutrient agar, potato-dextrose agar, and YMA, all adjusted to pH 3.5, it was concluded that the rate of yeast growth in YMA was the most favorable.

Stool samples were obtained primarily from two sources. Stools from individuals, not using any form of therapeutic drugs but confined to a nursing home for reasons other than infectious medical problems, were used. In addition, stools from healthy university student volunteers were obtained for study. One to four "wet" grams of human feces were suspended in sterile phosphate buffered water (pH 7) to yield a 1 to 10 percent suspension and macerated with glass beads on a reciprocal shaker until the samples were homogeneously suspended. Serial tenfold dilutions were made aseptically. Again, pour plates using YMA-acid media and diluted stool samples were made; the yeast colonies present after 3 to 5 days incubation at room termperature and 37°C were counted.

Various samples of wastewater were obtained at the East Side Treatment Plant operated by the Urbana and Champaign Sanitary District. The majority of wastewater samples, obtained at the plant, were initially filtered through several layers of cheesecloth at the time of sampling in the field and stored at 5°C for not more than 5 hr before being examined. In addition, in order to determine the effect of sample preparation on the apparent densities of organisms present in wastewater, alternate sample treatment methods were compared to cheesecloth filtration. Samples were taken without filtration directly to the laboratory and a portion of each sample was filtered through several layers of cheesecloth and vigorously shaken. The remaining portion of the sample was divided into two parts, one of which was homogenized by blending for about 30 sec while the other was vigorously shaken approximately 25

times as recommended in *Standard Methods* (44) in order to break the bacterial clumps. The yeast densities for each sample preparation method were then determined and compared.

Enumeration of yeasts in wastewater was accomplished by the pour plate technique using replicate plates of YMA-acid media and diluted wastewater. The yeast colonies present after 3 to 14 days incubation at 20°C and 37°C were determined.

Development of Selective Techniques for Yeast Enumeration in Fecal Material and Wastewater

Since YMA-acid medium also permits mold growth, difficulty was experienced in counting discrete yeast colonies. Several means of eliminating interfering contaminants were investigated.

A mold inhibitor, sodium propionate, was added at a concentration of 0.25 percent to suppress mold growth. Sodium propionate was found to be not entirely satisfactory in this regard. An anaerobic incubation method with a parafin layer on YMA-acid medium was also evaluated. To eliminate occasional acidophilic bacterial growth on YMA-acid plates, an antibiotic, oxytetracycline (Terramycin, Chas. Pfizer Co.) was incorporated into the medium. Ten mℓ of a 0.1 percent solution, freshly prepared and sterilized by membrane filtration, was added to 100 mℓ of melted 45°C YMA-acid medium at pH 3.5.

In order to compare the relative presence of indicator organisms with "normal" wastewater and fecal organisms, a total plate count was made in addition to yeast enumeration by preparing duplicate nutrient agar pour plates at each of several dilutions and counting the colonies present after a minimum 2 days incubation at 37°C.

Enumeration of Acid-Fast Bacilli in Fecal Material and Wastewater

Samples of wastewater and stools for the determination of acid-fast bacilli densities were obtained and treated essentially the same as for yeasts as outlined above.

Development of Selective Techniques for Enumeration of Acid-Fast Bacilli in Fecal Material and Wastewater

A similar problem of contaminants as in the yeast studies was encountered when stools and wastewaters were examined for acid-fast bacteria. Three approaches were taken to develop selective techniques for enumeration of acid-fast bacilli. First, several methods of pretreating samples prior to culturing were examined as to their relative efficacy in removing

contaminants. A second approach was to use various selective media for acid-fast bacilli. To some of the selective media various chemical agents were added in order to suppress growth of contaminats. Lastly, a technique was developed for the acid-fast staining of membrane filters for positive identification and enumeration of acid-fast organisms. The selective methods were evaluated for known acid-fast bacilli obtained from wastewater prior to study of fecal and wastewater samples.

Methods for the Pretreatment of Samples

Since sputum samples have been routinely examined for the presence of acid-fast tubercle bacilli for many years, the selective technique applied to sputum was investigated. The alkaline sputum treatment method (48) using 1, 2, 6, and 10 percent NaOH treatment was examined with known cultures of acid-fast bacilli obtained from secondary effluents. Samples were contacted with alkali for various time periods and then neutralized with dilute HCl.

In addition, pretreatment of sample with quaternary ammonium compounds was evaluated to determine the ability of this pretreatment to suppress growth of contaminants and to assess the ability of acid-fast organisms to survive this treatment. Four quaternary ammonium compounds (QAC), Arquad T-2C-50, Armeen C, Armeen 12D, and Roccal, were first dissolved in phosphate buffer and a predetermined amount added to cell suspensions for exposure periods of either 5 or 10 min. These suspensions were centrifuged to remove the cells from the QAC; the cells were then resuspended in buffer and plated in Middlebrook and Cohn 7H10 agar. Armeen C and Armeen 12D dissolved more completely in 40 percent ethanol than in buffer; thus, further experiments with these QAC were carried out using 40 percent ethanol solutions.

Pretreatment of cultures containing both acid-fast bacteria as well as potentially interfering microorganisms with 5 percent oxalic acid for 30 min followed by neutralization with 4 percent NaOH was recommended by David (49). Evaluation of this pretreatment was undertaken first by treating Isolate Nos. 132 and 134 with oxalic acid-NaOH to determine their sensitivity. In order to evaluate oxalic acid-NaOH pretreatment for wastewater and fecal samples, contact time and the oxalic acid concentration were varied.

Development of Selective Media for Enumeration of Acid-Fast Bacilli

Middlebrook and Cohn 7H10 agar is generally considered to be a selective medium for acid-fast organisms. However, in highly contaminated specimens, such as wastewater and feces, the selectivity of this medium was not sufficient to overcome the growth of other, non-acid-fast organisms. Moreover, the use of oxalic acid-NaOH and the other pretreatment methods

was found not to be sufficient because a number of spore forming organisms were observed to survive treatment in wastewater and fecal samples. A satisfactory selective medium for acid-fast bacteria is necessary due to their low concentration in wastewater in comparison to contaminant organisms.

Incorporation of chemotherapeutic agents and various dyes into an enriched acid-fast bacteria medium was investigated. Two acid-fast bacilli, Isolate Nos. 132 and 134, and samples of secondary effluent were studied separately in Middlebrook and Cohn 7H10 agar containing penicillin at concentrations from 10 to 250 units per mℓ of medium as well as malachite green and Eosin Y at concentrations of 0.005 to 0.0075 percent. In addition, several other drugs were tested against the two acid-fast bacteria using sensitivity discs impregnated with various quantities of the drugs. Impregnated sensitivity discs were placed on pour plates and the presence or absence of a zone of inhibition was noted. Studies of the relative sensitivity of wastewater organisms and the two acid-fast bacilli to eight antibiotics (chloromycetin, neomycin, erythromycin, kanamycin, novobiocin, penicillin, streptomycin, and tetracycline with 2, 5, 10, or 30 mcg per disc) and eight sulfa drugs (sulfamethacypyridazine, elkosin, gantrisin, sulfadiazine, sulfamerazine, sulfa thiazole, thiosulfil and triple sulfa with 50 or 300 mcg per disc) were carried out to determine the usefulness of their incorporation into selective media using the sensitivity disc method. Additionally, five sulfa drugs (kynex, gantrisin, elkosin, sulfathiazole and sulfamethizole) were suspended in phosphate buffer, then later in 40 percent ethanol, and a predetermined amount was incorporated into Middlebrook and Cohn 7H10 agar. The resistance of the two acid-fast bacilli and activated sludge effluent organisms was examined separately and quantified in terms of survival at various drug concentrations as compared to control plates containing no antibiotics or sulfa drugs.

Since many selective culturing techniques are based on information generated from knowledge of the microbial metabolic pathways, a selective culturing medium for acid-fast bacilli using organic acids and other metabolites as the sole carbon source was investigated. Mineral salt medium (NaCl, 0.1 g; $CaCl_2$, 0.1 g; $FeCl_3$, 0.02 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $[NH_4]_2SO_4$, 0.7 g; KH_2PO_4, 1.0 g; agar, 15 g; distilled water, 1000 mℓ) containing a parafin hydrocarbon as the sole carbon source was evaluated as a selective medium for acid-fast bacilli. Additional carbon utilization studies, including 15 carbon sources; acetate, benzoate, pyruvate, lactate, propionate, malate, oxalate, citrate, tartrate, glycerol, succinate, glucose, maltose, lactose, and mannose, were carried out with the acid-fast cultures. A buffered mineral medium containing the single carbon source under investigation was used to culture the acid-fast bacilli. Pour plates were inoculated with stock cultures of the acid-fast isolates and growth noted during a 2 weeks incubation period at 37°C.

Development of an Acid-Fast Staining Procedure for Membrane Filter Enumeration of Acid-Fast Bacilli

Another approach taken to the problem of selectively enumerating acid-fast bacilli in wastewater was the development of a membrane filtration technique. The general procedure for membrane filtration of bacteria in wastewater, outlined in *Standard Methods* (44), was modified by using an enrichment broth and medium specific for acid-fast bacilli, e.g., Middlebrook and Cohn 7H10 agar. The plates containing the membranes were incubated in a humidified incubator at 37°C until the colonies were visible but not so large that they were easily washed off the filter when subjected to the staining procedure. A filter was removed from the agar with a forceps and carefully dried above a flame so as to prevent charring the filter. The filters (Type HA Millipore filters, Millipore Corp., Bedford, Mass.) were stained by a modified acid-fast staining procedure. The solution used in the procedure included 0.5 mℓ Brook's carbol fuchsin-dimethylsulfoxide stain in 20 mℓ water and 0.5 mℓ Brook's malachite green stain containing the decolorizer (acid-alcohol) in 20 mℓ water; both reagents were obtained from Curtin Scientific (St. Louis, Missouri). The two dye solutions, water for washing the filters, and absolute ethanol were placed in regular sized petri dishes. The filter was immersed in the carbol fuchsin solution for 30 sec and then rinsed completely in water by gently swishing the filter in water with the forceps. The membrane was decolorized by immersion in ethanol and stained in the malachite green solution for 30 sec. After rinsing in water, the membrane was allowed to dry and colonies were observed under a dissecting microscope at a magnification of 50 X.

Since no single selective method was completely satisfactory for the routine isolation of acid-fast bacteria from mixed microbial populations such as in wastewaters and feces, methods combining the most satisfactory pretreatment and culturing techniques of those studies were evaluated. Since the samples should be homogenous, large clumps were dispersed by the use of a blender. Samples were then centrifuged to further separate particulates from the liquid suspension. The centrifuged particles were resuspended in sterile phosphate buffer and the procedure repeated if significant quantities of solid particles were observed. The homogeneous liquid suspension was then treated with 2.5 percent oxalic acid for 5 to 10 min, followed by neutralization with 2 percent NaOH. The sample was then diluted with sterile phosphate buffer prior to application of the membrane filter technique. After 2 to 3 days incubation on a selective medium or when visible colonies were evident, the filter was stained with the modified acid-fast staining procedure and examined for pink colonies indicating positive acid-fast bacilli.

SECTION V

RESULTS AND DISCUSSION

ISOLATES EXAMINED FOR CHLORINE RESISTANCE

Pure cultures of 135 isolates were collected for chlorination resistance studies. Although most of these organisms were isolated from secondary effluents, three bacterial cultures were obtained from chlorinated tap water and thirteen pure cultures of known species were obtained from the Department of Microbiology, University of Illinois. The known stock cultures included the genera: *Flavobacterium*, *Pseudomonas*, *Serratia*, *Micrococcus*, *Sarcina*, *Chromobacterium*, *Staphylococcus*, *Micromonospora*, and *Streptomyces*. Isolates were also established by subculturing from selected colonies surviving on the assay plates in the preliminary chlorine screening tests. It was postulated that these survivors might have greater resistance to chlorine than the parental isolates.

Conditions of isolation and properties of representative isolates are given in Table 1. For isolation, this includes the source of each isolate, the amount of chlorine added to the effluent and, in some cases, the free and total chlorine residuals remaining after 5 or 30 min, the medium, and temperature of incubation. The properties reported in Table 1 are morphology, and Gram reaction. Many of the bacteria found after chlorination of secondary effluents were Gram positive spore forming rods. Only a few of these were retained for further study because of the dependence of their spore forming properties on environmental conditions which makes them generally unsuitable as indicator organisms. Many different morphological types of organisms were isolated, primarily at incubation temperatures from 20° to 37°C. Isolates included both Gram positive and negative rods, cocci, and mycelia with white, orange and yellow pigments. Organisms were found when as much as 126 mg/ℓ of chlorine was added to an effluent sample to give 25 mg/ℓ total and 17 mg/ℓ free chlorine residual. Acid-fast bacteria, yeasts and actinomycetes were the most unusual isolates. The isolation of organisms was conducted over a sufficiently long period to account for any seasonal variation in the wastewater microbial population. Considering the number of isolation experiments and the period of examination it is believed that the isolates obtained were representative of those that might be commonly found in effluents.

FURTHER EXAMINATION OF ISOLATES FROM CHLORINATED WASTEWATER

Although the isolates survived various degrees of secondary effluent chlorination (Table 1), most of the organisms isolated were too sensitive to chlorine to be useful bioindicators, according to the criteria selected for this study; that is, some degree of survival at concentrations and contact times up to 1 mg/ℓ free residual chlorine for 30 min.

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Table 1

Conditions of Isolation and Properties of Representative Isolates

	ISOLATION						PROPERTIES	
Isolate No.	Source[1]	Chlorine Added mg/ℓ	Free Residual mg/ℓ	Total Residual mg/ℓ	Media[2]	Temp. °C	Morphology	Gram Reaction
5	AS	30.6			NA	20, 37	rust, rods, spores	+
27	AS	42.2			NA	37	yellow rods	-
30	AS	15.2			NA	37	oval budding yeast	+
32	AS	15.2			NA	37	orange mycelium	+
37	AS	44.9			NA	20	oval cocci	-
62	AS	63.0		31.5	SA	37	yellow rods	-
67	AS	113.4	2.0		NA	20	yellow rods	-
70	AS	113.4	2.0		ST	37	curved rods	-
75	TF	126.0	17.0	25.0	NA	37	short rods	-
82	TF	25.2		4.6	NA	20	round budding yeast	+
87	TF	25.2		4.6	TS	20	gold, rods, spores	+
88	TF	100.8	4.3	19.0	ST	37	yeast, true mycelium	+

Table 1 (continued)

	ISOLATION					PROPERTIES	
Isolate No.	Source[1]	Chlorine Added mg/ℓ	Free Residual mg/ℓ	Total Residual mg/ℓ	Media[2]	Temp. °C	Morphology	Gram Reaction
90	TF	12.6		6.0	NA	37	granular rods	-
106	TF	37.8		24.0	NA	37	orange mycelium	+
114	OP	30.0		22.7	NA (pH 9)	37	large cocci in yellow packets	+
124	AS	30.0		23.0	7H10	25	orange rods	-
128	TF	30.0		25.0	NA	10	yellow coccobacilli	-
132	TF	30.0		25.0	7H10	25	yellow rods, acid fast	
134	OP	30.0		22.7	7H10	25	thin rods, acid fast	

[1]Effluents – AS, Activated Sludge; TF, Trickling Filter; OP, Oxidation Pond

[2]Media – NA, Nutrient Agar; SA, Succinate Agar; TS,Trypticase-soy Agar; ST, Starch; 7H10, Middlebrook and Cohn

There was no significant correlation found between the chlorine resistance of the isolates as determined in chlorine demand free water and the chlorine dosage used in the isolation experiments. This may be ascribed to organism protection by clumping and by extraneous materials in the chlorinated effluent. None of the tap water isolates nor the known stock cultures tested were sufficiently chlorine-resistant to satisfy the initial screening criteria.

Excluding spore-forming bacilli, the isolates which showed the most chlorine resistance were yeasts and acid-fast bacilli which were all able to grow on nutrient agar as well as on several other types of media at 37°C. The general shape of the inactivation curves followed a pattern frequently reported by various authors, with a lag phase followed by an increasing rate which finally leveled off. However, the length of each section of the curve as well as its rate varied with the chlorine concentration, the organism being studied, and other undefined experimental conditions, e.g., those relating to the growth of the culture in the nutrient broth.

The first promising isolate found was No. 30, an oval-shaped, asporogeneous, budding yeast that forms pseudomycelia. Curves indicating its survival to chlorination (Figure 1) show that 0.08 percent of the cells survived a free chlorine residual of 1.0 mg/ℓ for 20 min while survival after 30 min exposure at 0.1 mg/ℓ free residual chlorine was greater than 80 percent.

In studying Isolate No. 30, one of the colonies which appeared on the assay plate following exposure to 1.0 mg/ℓ free residual chlorine for 20 min was subcultured as Isolate No. 30A. Studies on No. 30A showed that it was more resistant to chlorine than the parent culture, No. 30, with nearly 0.2 percent of the initial inoculum surviving a free chlorine residual of 1.0 mg/ℓ for 30 min (Figure 2). However, in later experiments the chlorine resistance of No. 30A appeared to decrease somewhat, approaching that of Isolate No. 30. This may have been due to an unstable mutation to higher resistance or to some function of cultivation of the organism.

A spherical budding yeast, Isolate No. 82, was also more resistant to chlorine than most of the bacteria examined (Figure 3); however, because its chlorine resistance was much lower than that of No. 30, organism No. 82 was not studied in any further detail. Only about 2 percent of the cells survived a free chlorine residual of 1.0 mg/ℓ after a 5 min exposure.

Isolate No. 132 is an acid-fast yellow pigmented rod which showed promising chlorine resistance. Figure 4 shows 63 percent survival after 30 min contact of 0.1 mg/ℓ free residual chlorine and 0.05 percent survival after 15 min of 1.0 mg/ℓ. Although always remaining at quite a high level, the resistance of this organism was more variable than that of the other isolates in comparing different experiments.

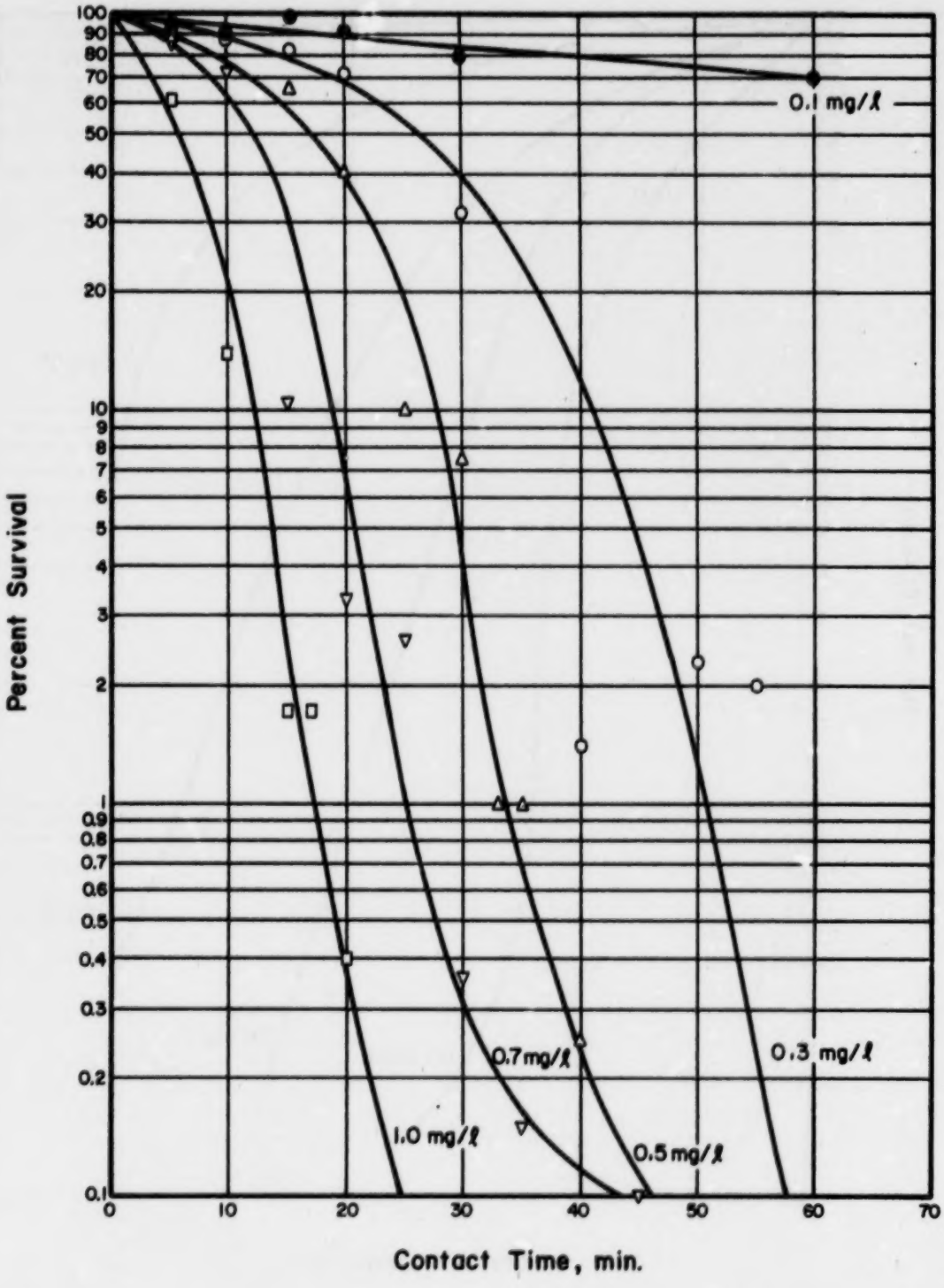

FIG. 1 RESPONSE OF ISOLATE NO. 30 TO FREE CHLORINE RESIDUALS RANGING FROM 0.1 TO 1.0 mg/ℓ

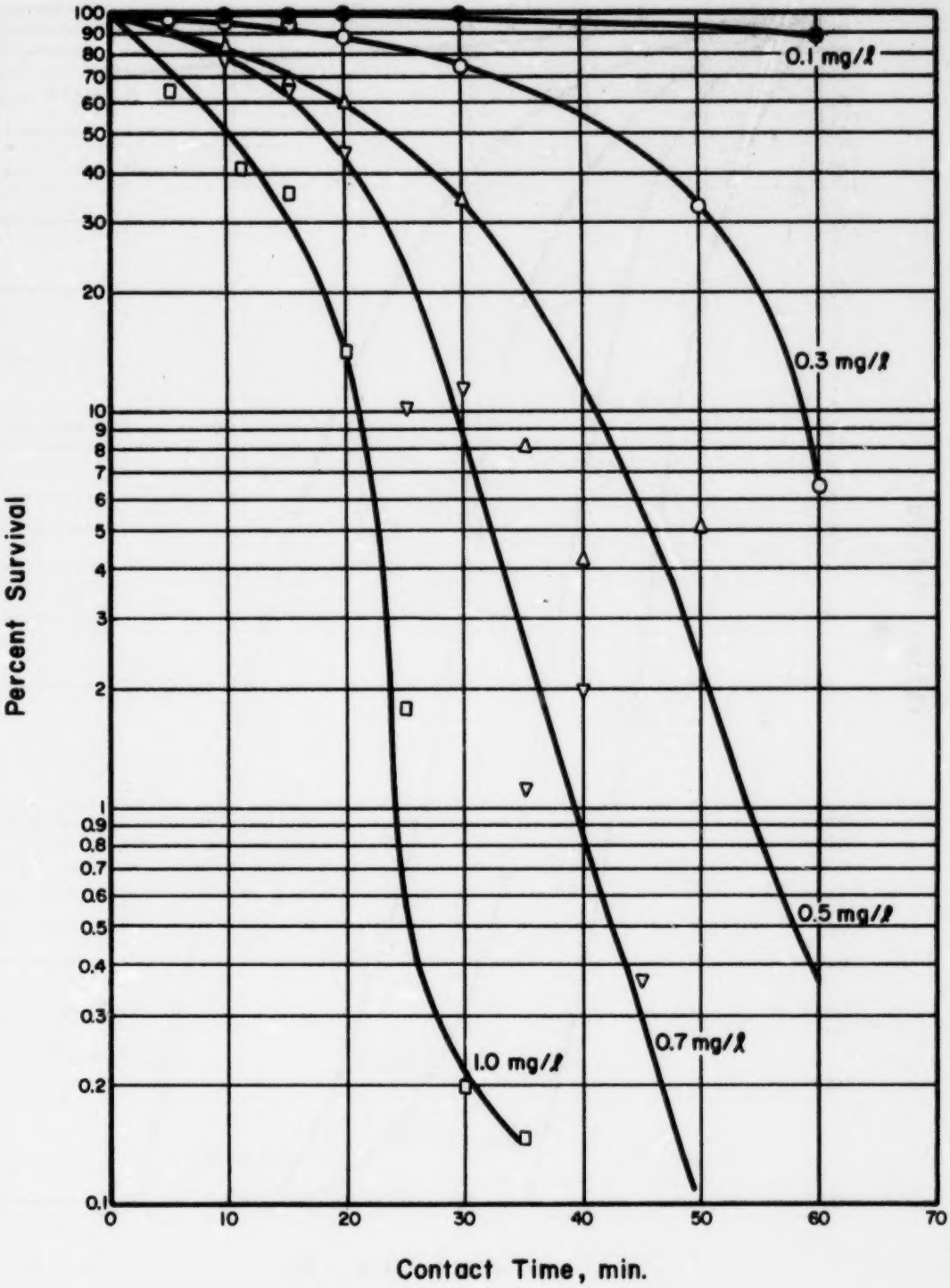

FIG. 2 RESPONSE OF ISOLATE NO. 30A TO FREE CHLORINE RESIDUALS RANGING FROM 0.1 TO 1.0 mg/ℓ

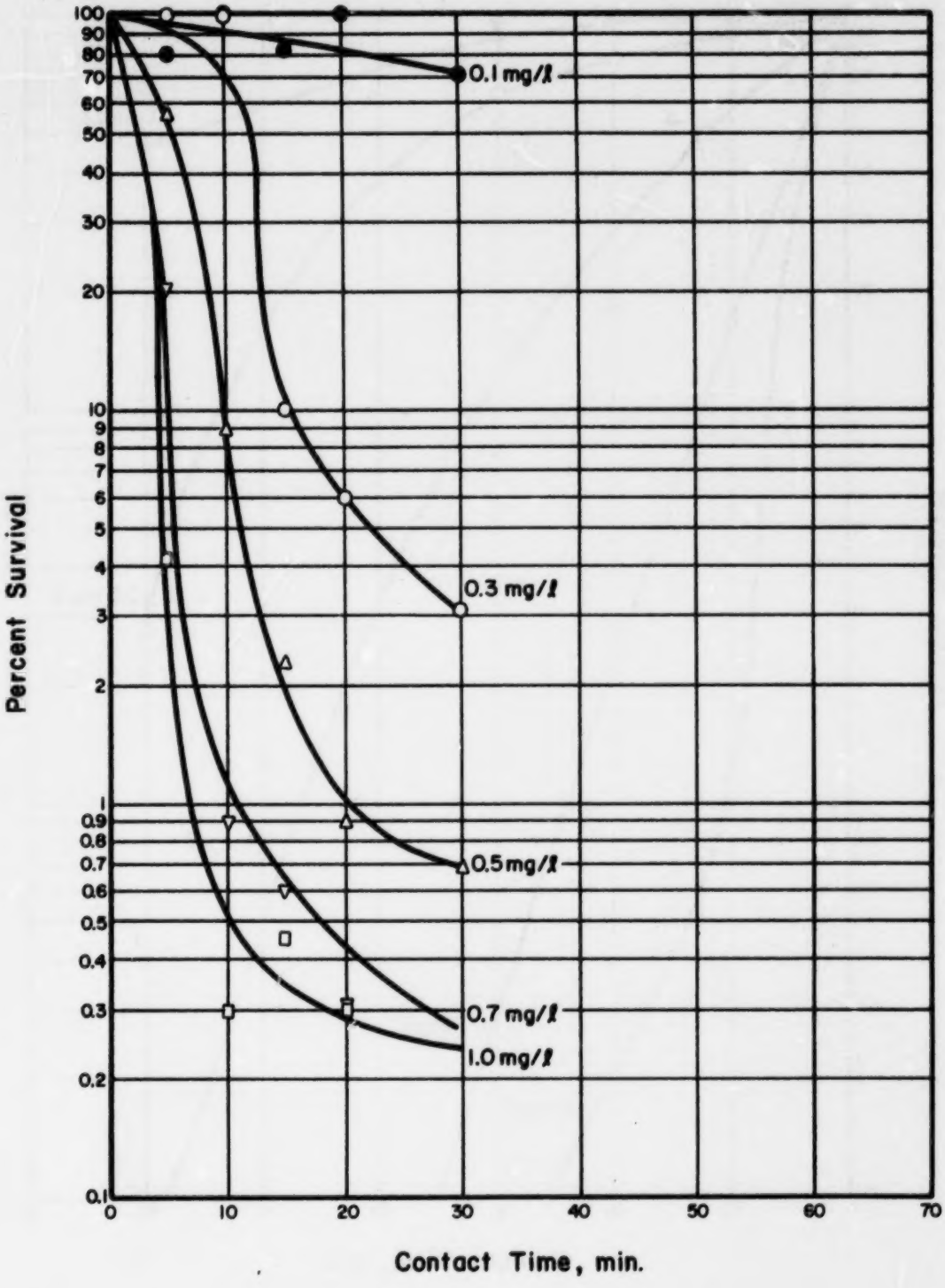

FIG. 3 RESPONSE OF ISOLATE NO. 82 TO FREE CHLORINE RESIDUALS RANGING FROM 0.1 TO 1.0 mg/ℓ

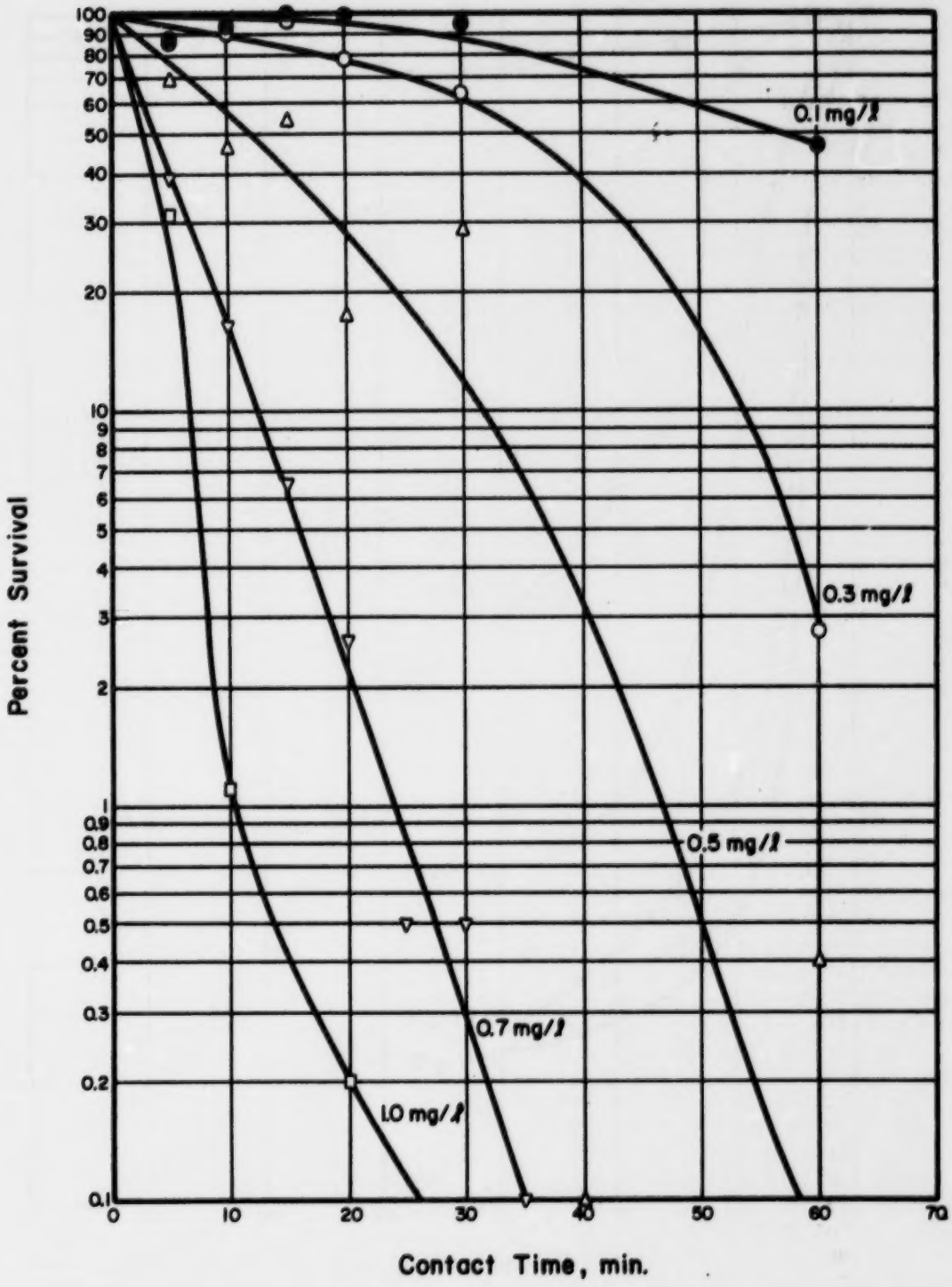

FIG. 4 RESPONSE OF ISOLATE NO. 132 TO FREE CHLORINE RESIDUALS RANGING FROM 0.1 TO 1.0 mg/l

The most resistant organism isolated was No. 134, another acid-fast bacillus. To obtain satisfactory survival curves for Isolate No. 134, it was necessary to use higher dosages of chlorine than those used in experiments with the other isolates (Figure 5). The lowest concentration used was 0.5 mg/ℓ and 2.0 mg/ℓ the highest, although 1.5 mg/ℓ was the highest dosage applied in most experiments. In one test, 2 percent of the cells survived a free chlorine residual of 2.0 mg/ℓ for 67 min.

The free chlorine residuals and contact times necessary to achieve 99.9 percent kill of the four test organisms have been extrapolated from the data in Figures 1, 2, 3, 4, and 5 and are presented for comparison with data compiled by Berg (50) from chlorination studies on *E. coli*, adenovirus, poliovirus 1, and Coxsackie virus A2 (Figure 6). The isolates collected from wastewater in the current study show a definite trend toward increased chlorine resistance over that of the traditional coliform indicator. Isolate Nos. 30, 30A, and 132 show resistance comparable to or greater than that reported for resistant pathogens such as Coxsackie A2 virus. Isolate No. 134 shows a significantly greater resistance than the pathogens. It must be pointed out that there is an inherent danger in comparing data presented by several investigators collected under possibly dissimilar conditions. Nevertheless, the data do suggest that it is possible to obtain vegetative bacteria with sufficient chlorine resistance to make them suitable as indicators of the efficiency of chlorination in destroying resistant pathogens.

Examination of Figure 6 might suggest that Isolate No. 134 could be too chlorine resistant to be useful as an indicator organism. This is not the case for two reasons. First, it is desirable that an indicator organism provide a reasonable margin of safety in indicating the efficiency of chlorination in destroying pathogens. Secondly, the pathogens cited in Figure 6 represent only a few of the chlorine resistant pathogens found in wastewater and, therefore, might not be representative of the most chlorine resistant pathogens present in wastewater. Indeed, Liu (4) recently showed, in a study of 20 viral pathogens, that resistance depended on the type of data used to judge resistance. For example, the most resistant viruses, using percent survival at 5 min contact with 0.5 mg/ℓ free chlorine as a judgment criterion, were not the same as those found to require the longest time to reach 99.99 percent destruction, assuming first order kinetics (4). In either case, Isolate Nos. 134 and 30A show more resistance to chlorine than the most resistant viral pathogens studied by Liu. Isolate Nos. 30 and 132 show superior survival at 5 min and a similar contact time necessary to achieve a 99.9 percent destruction. It should be pointed out that the experimental conditions used by Liu were different from those used in studying the organisms isolated in the current study. Direct comparison of pathogens and the indicator organism under the same conditions is the only exact manner in which relative resistance to chlorine may be established.

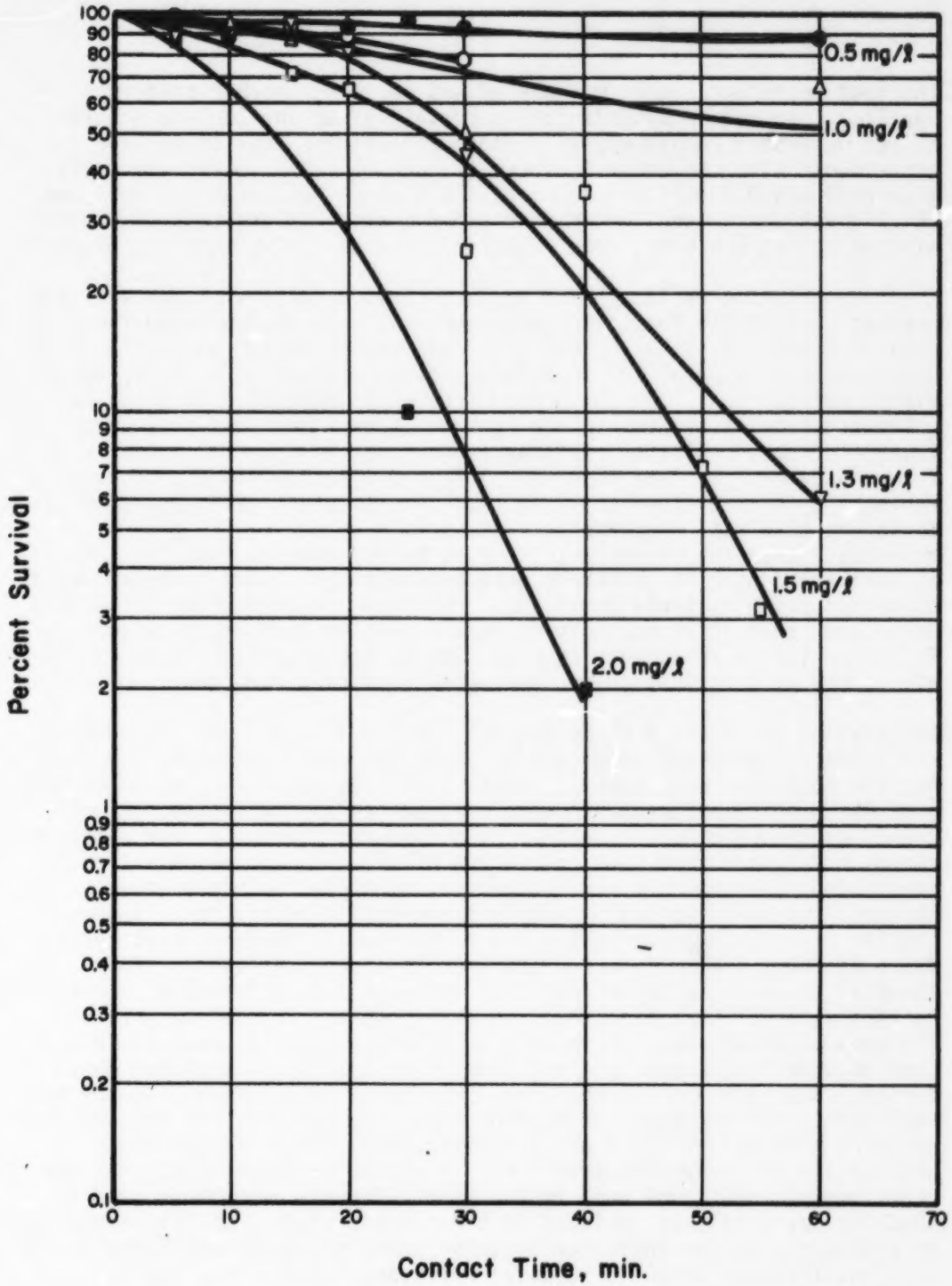

FIG. 5 RESPONSE OF ISOLATE NO. 134 TO FREE CHLORINE RESIDUALS RANGING FROM 0.5 TO 2.0 mg/ℓ

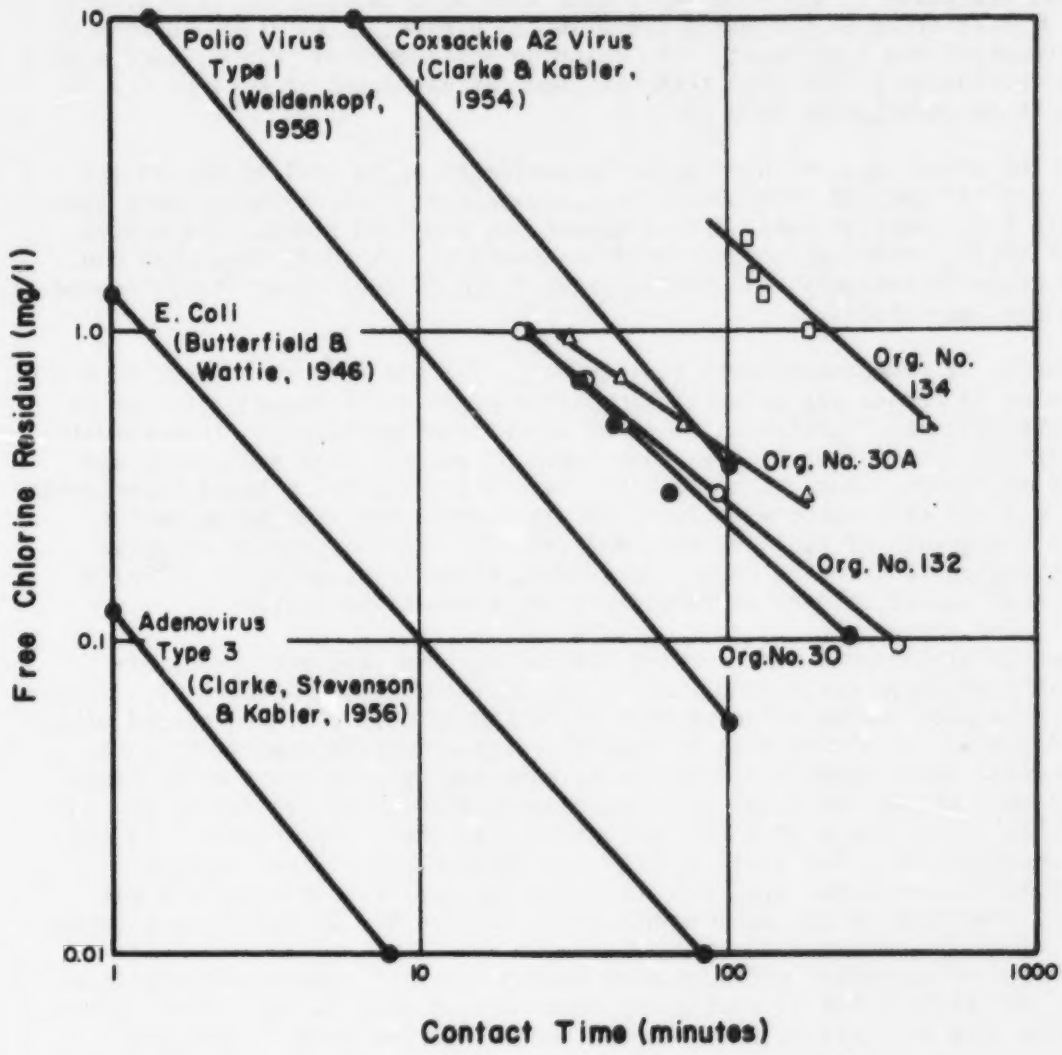

FIG. 6 FREE CHLORINE RESIDUALS AND CONTACT TIMES NECESSARY FOR 99.9 PERCENT KILL FOR ISOLATES NO. 134, NO. 30A, NO. 132 and NO. 30 AS WELL AS FOR ORGANISMS COMPILED BY BERG (50)

The results of screening *E. coli* ATCC-11229 with free residual chlorine concentrations of 0.01 to 0.04 mg/ℓ (Figure 7) showed acceptable agreement with similar data collected by Butterfield *et al.* (42) for the same *E. coli* strain. The free chlorine residuals obtained by Butterfield *et al.* (45) decreased in the reaction flasks from 0.03 to 0.02 and from 0.02 to 0.01 mg/ℓ after 60 min while those in the present study remained constant throughout the experiment. The chlorine resistance of this *E. coli* strain is considerably less than that observed for pathogens studied by Liu (4) or those compiled by Berg (50).

In the mixed culture chlorination experiments using Isolate No. 30 and *E. coli* ATCC-11229, the percentage survivals of both organisms were less than those observed when each organism was examined alone. The reason for this discrepancy was not readily apparent. It could have been due to organism antagonism in the reaction flask or even growth interference in the agar plates.

Results of experiments with Isolate Nos. 132 and 134 using Tween 80 and mixing to remove the effects of possible clumping of these organisms on their chlorine resistance showed no significant differences from results obtained in previous studies when Tween 80 was not used and mixing was not employed. Thus, clumping either was not a factor in these experiments or was not eliminated when Tween 80 was used in nutrient broth medium for the growth of Isolate Nos. 132 and 134. Examination of organism resistance to chlorine under static conditions appeared to be as valid as when the tests were performed with mixed reaction systems.

Several experiments were carried out to determine the efficiency of chlorination in destroying Isolate No. 30 in wastewater (Table 2). Samples of activated sludge effluent were collected in July 1971 and seeded with Isolate No. 30 and studied in controlled chlorination experiments. In general, even though the chlorine dosages employed in these experiments did not satisfy the total chlorine demand, Isolate No. 30 failed to consistently survive even 5 min contact in 2 of the 3 experiments. In the experiment where the yeast isolate did exhibit significant survival (7/20/71), the total available chlorine residual at 5 min contact was less than that in the experiments where Isolate No. 30 failed to survive.

Samples of secondary effluent were diluted tenfold, seeded with Isolate No. 30, and studied in controlled chlorination experiments. These experiments were designed to allow use of lower chlorine doses to achieve a desired total chlorine residual and to provide a situation similar to that which might be seen in AWT type effluents. However, the results may differ from those obtained in chlorinating actual AWT effluents since the concentrations and types of organic matter and nitrogenous compounds in such effluents could differ from those in diluted secondary wastewater effluents. In addition, the concentration of suspended matter in diluted secondary effluent may not be truly representative of AWT effluents. Survival curves obtained for diluted wastewater samples are presented

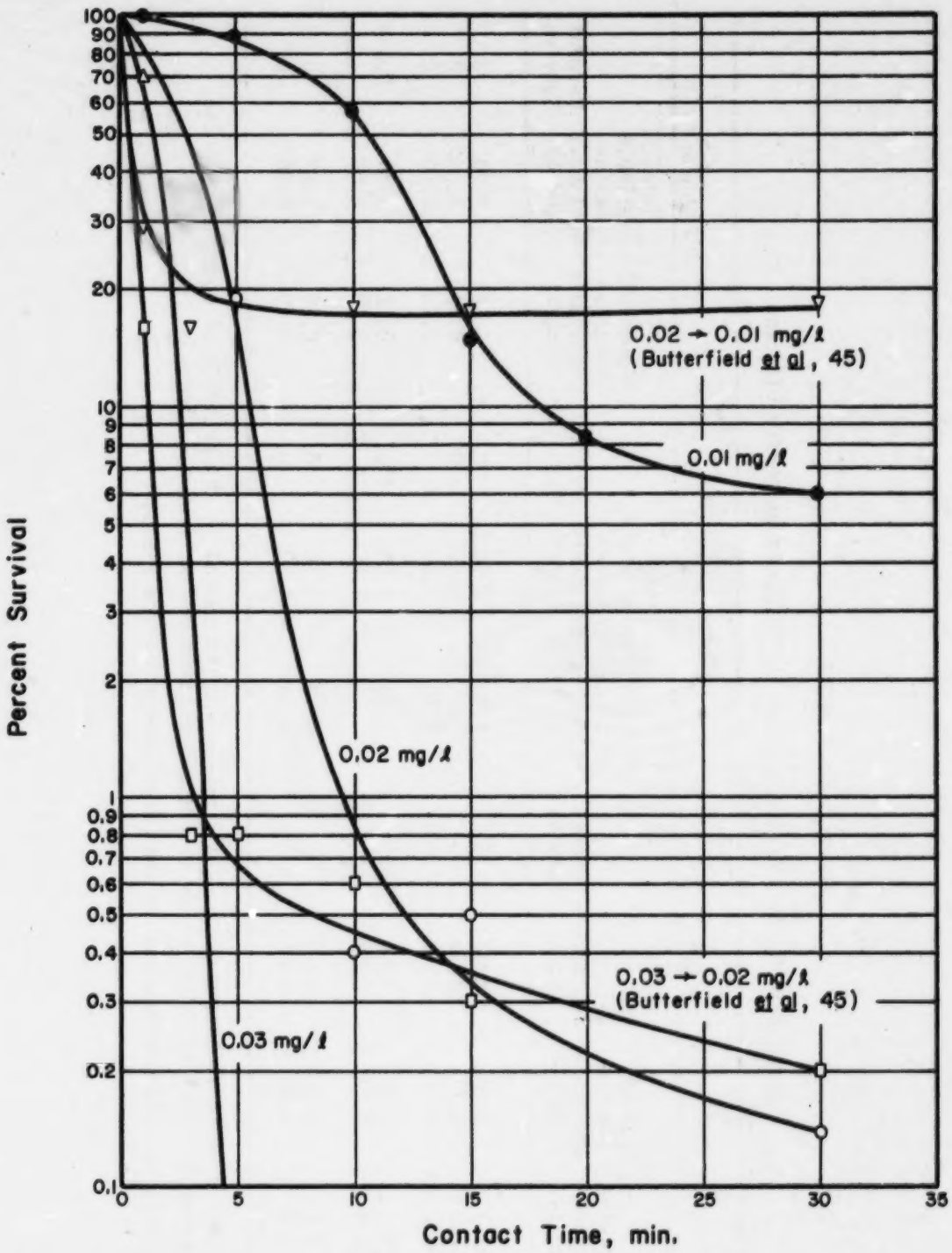

FIG. 7 COMPARISON OF RESPONSE OF E. coli ATCC-11229 TO FREE CHLORINE WITH DATA FROM BUTTERFIELD et al (45)

TABLE 2

Chlorination Study of Isolate No. 30 Seeded in Secondary Effluent

Exp. Date	Chlorine Dosage (mg/ℓ)	Contact Time (min) 5 min: Apparent[a] Chlorine Residuals (mg/ℓ)	5 min: Total[b] Available Chlorine Residuals (mg/ℓ)	5 min: Percent Survival	15 min: Apparent Chlorine Residuals (mg/ℓ)	15 min: Total Available Chlorine Residuals (mg/ℓ)	15 min: Percent Survival	30 min: Apparent Chlorine Residuals (mg/ℓ)	30 min: Total Available Chlorine Residuals (mg/ℓ)	30 min: Percent Survival
1971 7/13	47.1	1.60	4.60	0	1.30	2.60	0	1.00	2.20	0
	47.5	0.73	8.10	0	0.25	4.60	0	0.17	4.00	0
	48.0	1.10	9.30	0	0.18	7.30	0	0.18	0.90	0
7/20	33.8	0.10	2.56	73.1	0.10	2.54	11.7	0.12	2.49	0.5
	34.4	0.12	2.69	67.7	0.12	2.65	14.9	0.10	2.50	1.1
7/23	4.8	1.02	3.46	0	0.40	1.74	0	0.25	1.22	0
	5.2	1.35	3.40	0	0.70	1.50	0	0.60	0.96	0

[a]Free chlorine residual determined within 2 min by the DPD method in the presence of combined chlorine; interference by combined chlorine may be present and measured as free chlorine

[b]Total available chlorine measured by the DPD-potassium iodide method

in Figure 8. Survival (Isolate No. 30) was measurable at 30 min contact but varied widely with survivals of 0.6 and 49.9 percent for experiment with 2.75 and 1.4 mg/ℓ total available chlorine, respectively, at 30 min. Although not shown in Figure 8, survival amounted to 0.01 percent with a total chlorine residual of 4.80 mg/ℓ and a 30 min contact time.

The low survival of Isolate No. 30 in the data of Table 2 and Figure 8 is not surprising when one considers the high chloramine concentrations involved in these studies. High chloramine concentrations can be an effective disinfectant with the contact periods employed with these data. In addition, a transitory free chlorine residual most likely will be present immediately after the addition of chlorine and prior to the reaction of chlorine with wastewater organic and inorganic amines. At the high chlorine dosages employed in these experiments a considerable level of free chlorine would be present in the reaction flask for a period of time.

ENUMERATION OF YEASTS IN FECAL MATERIAL AND WASTEWATER

Fifty-four fecal samples from healthy human volunteers were examined for total plate count bacteria on nutrient agar and yeast densities using acidified nutrient media, i.e. nutrient agar or YMA. The data (Table 3) indicates little or no correlation between the number of organisms determined by total plate count and the yeasts present in feces. The majority of the stool samples, at both incubation temperatures (room temperature, approximately 25°C, and 37°C), showed 0 to 50 yeasts/g on a wet basis (Table 4). In samples numbered 24 to 54, one-fifth of the stools examined showed yeast densities in the high range of 1001 to 10,000/g of feces. These latter stool samples were tested using a more concentrated suspension of fecal material than used in samples 1 to 23. This appears to have improved sensitivity. Approximately 41 percent of samples 1 to 23 showed the presence of yeasts, while 69 percent of samples 24 to 54 showed yeasts with incubation at 37°C. Use of room temperature for incubation of samples also improved results. Over 80 percent of the samples were positive when plated on pour plates incubated at room temperature (approximately 25°C). When the arithmetic means for room temperature and 37°C data are compared for those samples where paired data exists, room temperature data indicate an advantage over 37°C incubation by 1,481 to 1,360 yeasts/g of feces. Of the 28 possible sets of data paired, 16 show greater values for room temperature incubation and only six for 37°C, with six giving an equal count at both temperatures of incubation. It has been reported that the pH of the medium, its composition and the temperature of incubation have a significant effect on the enumeration of yeasts. With respect to temperature, 21°C or 25°C has been suggested to be optimum (51); this is in agreement with the findings of this study. Therefore, room temperature with YMA acid medium at 10 percent or higher concentration of fecal material seems to be the technique of choice for examination of stool samples for yeasts. From this limited study of 54 fecal samples it may be concluded that an

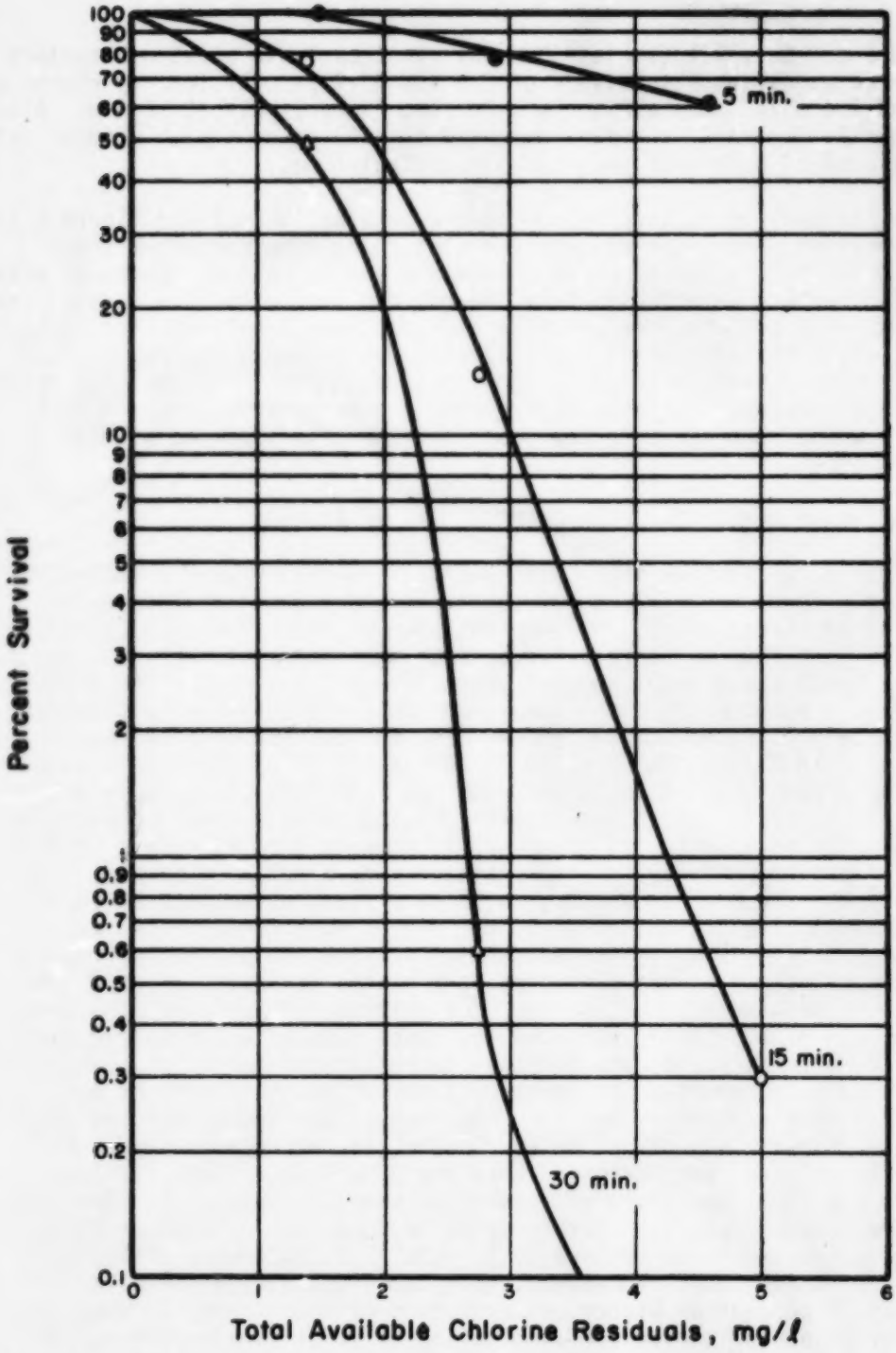

FIG. 8 CHLORINATION STUDY OF ISOLATE NO. 30 SEEDED IN DILUTED SECONDARY EFFLUENT

TABLE 3

Examination of Fecal Samples for Total Plate Count and Yeasts

Sample Number	Total[1] Plate Count 37°C	Yeasts[2] 25°C	Yeasts[2] 37°C	Sample Number	Total[1] Plate Count 37°C	Yeasts[2] 25°C	Yeasts[2] 37°C	Sample Number	Total[1] Plate Count 37°C	Yeasts[2] 25°C	Yeasts[2] 37°C
1	33		0	19	1.8		0	37	62	18	15
2	15		0	20	13		0	38	11	0	1
3	36		0	21	8		200	39	1.7	11,000	10,000
4	18		4,000	22	29		0	40	1.6	6	5
5	1.6		-	23	320		150	41	2	1	0
6	5.5		100	24	280	30	-	42	18	2,400	2,230
7	13		0	25	2	75	38	43	55	72	68
8	102		0	26	170	620	440	44	5.2	36	41
9	67		400	27	40	12	12	45	800	2	0
10	35		0	28	65	0	0	46	140	12	0
11	0.3		0	29	2.7	140	150	47	470	11,200	10,000
12	0.25		0	30	61	0	0	48	400	5,900	4,300
13	2		1,150	31	3.6	1,040	1,120	49	480	5	290
14	9		250	32	6.6	1,160	-	50	170	10	0
15	1.2		0	33	3.9	0	0	51	230	-	13,500
16	1.6		0	34	38	3	0	52	1.7	5,000	5,000
17	1.8		250	35	35	0	0	53	6.7	2,310	2,800
18	4.1		100	36	25	25	19	54	330	1,610	1,600

[1] Number x 10^6 per wet gram feces

[2] Number per wet gram feces; samples 1 and 2 were plated on acidified potato dextrose agar; samples 3, 7 and 8 were plated on acidified nutrient agar; all others were plated on acidified YMA; samples 1-23 were macerated at 1 percent concentrations in phosphate buffer while 4 to 10 percent suspensions were used for samples 24-54.

TABLE 4

Statistical Analysis of Fecal Yeast Densities

A.

Frequency Distribution of Fecal Yeast Densities[1]

Yeast per gram feces	Samples 1 - 23[2] 37°C	Samples 24 - 54[3] Room Temp.[4]	37°C
0	59.1	16.7	31.0
1-50	0.0	40.0	24.3
51-100	9.1	6.7	3.5
101-500	22.7	3.3	10.3
501-1,000	0.0	3.3	0.0
1,001-10,000	9.1	23.3	20.6
>10,000	0.0	6.7	10.3

B.

Statistical Values for Fecal Yeast Densities

	Samples 1 - 23 37°C	Samples 24 - 54 Room Temp.[4]	37°C	All Samples 37°C
Mean	300	1420	1720	1141
Median	0.3	27.5	37.8	15
Percent Positive	40.9	83.3	69.0	52.9

[1]Values are percent of total number of samples.

[2]Includes 1 percent dilution of fecal material and incubation using either nutrient agar, potato dextrose agar, or YMA media.

[3]Includes 4 to 10 percent dilution of fecal material and incubation using only YMA medium (pH 3.5-3.9).

[4]Approximately 25°C.

encouragingly high percentage of the population show yeasts in their stools, and in concentrations up to 10^4/g of fecal material.

In research complementary to the objectives of this study but not supported directly by this grant, Panswad (52) studied yeast densities in raw wastewater and wastewater effluents of the East Side Treatment Plant of the Urbana and Champaign Sanitary District. He found that there was a variation in the number of yeasts detected at 20° and 37°C. After 3 days of incubation more yeasts were observed by colony formation at 37°C than at 20°C in general but, as incubation time increased, this observation was reversed, i.e. more yeast colonies were detected at 20°C. Mossel *et al.* (51) also observed that incubation at 20° to 22°C gave much higher yeast counts. Based upon visual inspection, more rapid colony development of yeasts occurred at 37°C for no growth was evident at the lower temperature (20°C) until after 3 days of incubation.

A mold inhibitor, sodium propionate, added into YMA medium was found to inhibit yeasts as well as interferring organisms in wastewater and was not useful for that reason. When 100 ug/mℓ oxytetracycline was incorporated into the medium to inhibit acidophilic bacteria, some degree of yeast suppression was indicated by a lower yeast colony count. Thus, no adjustment other than lowering the pH value of YMA medium was used to provide a selective technique for yeast enumeration.

The yeast densities found in the Urbana and Champaign Sanitary District wastewater samples are presented in Tables 5, 6, 7, 8, 9, 10, and 11. It should be noted that this treatment plant is somewhat unique in that the raw wastewater flow is split and a portion treated by the activated sludge process and part by trickling filters. This permits direct comparison of two secondary treatment processes treating the same raw waste. These data are classified to show the effects of pretreating the samples by homogenization (shaking or blending) with that of filtration plus shaking.

The pretreating of samples was designed to disrupt any clumps present in the samples and, therefore, release any yeasts imbedded in clumps so that these yeasts could be enumerated. Filtering through cheesecloth, a common practice in studying wastewater microorganisms, would remove many clumps and any yeasts associated with them, while vigorously shaking the samples 25 times or blending for 30 sec should break up the clumps. The pretreatment appeared to have its greatest effect on raw wastewater and the least effect on activated sludge effluent samples. Using data for 6 days of incubation, the ratios between filtered and shaken, shaken only, and blended samples were 1:5:4, 1:0.9:1, and 1:2.1:1.8 for raw sewage, activated sludge, and trickling filter effluents, respectively. Apparently many yeasts are associated with clumps affected by the pretreatment in raw wastewater, fewer in trickling filter effluents, and very few, if any, in activated sludge effluents.

TABLE 5

Yeast Densities in Raw Municipal Wastewater
(Acidified YMA Medium at 20°C)

Date	Avg. Wastewater Flow (mgd)	Yeast Count per mℓ								
		Filtered and Shaken			Shaken			Blended		
		Incubation, days			Incubation, days			Incubation, days		
		4	6	10	4	6	10	4	6	10
10/05/71	11.9	*								
10/12/71	9.5	370	500	*						
10/21/71	8.8	290	400	500						
10/27/71	9.1	300	450	630						
12/07/71	13.1		80	100						
12/14/71	14.5	930	1500	*	3500	4000	4300	2300	3000	3200
12/16/71	16.4	240	290	300	800	1000	1100	400	500	600
12/17/71	16.4	260	340	450	500	800	1000	650	1000	1250
Average Value		250	400	500	1400	1900	2100	1100	1500	1700

*Enumeration was not possible because of the spreading of mold growth on the medium surface.

TABLE 6

Yeast Densities in Activated Sludge Effluent
(on Acidified YMA Medium at 20°C)

Date	Avg. Wastewater Flow (mgd)	Yeast Count per mℓ								
		Filtered and Shaken			Shaken			Blended		
		Incubation, days			Incubation, days			Incubation, days		
		4	6	10	4	6	10	4	6	10
10/05/71	11.9	201	*							
10/12/71	9.5	95	130	210						
10/21/71	8.8	280	310	400						
10/27/71	9.7	30	80	150						
12/07/71	13.1		40	60						
12/14/71	14.5	180	400	630	220	300	*	175	300	600
12/16/71	16.4	120	175	215	120	150	160	150	170	215
12/17/71	16.4	95	140	200	100	140	190	100	180	350
Average Value		180	220	310	147	200	-	140	220	370

*Enumeration was not possible because of the spreading of mold growth on the medium surface.

TABLE 7

Yeast Densities in Trickling Filter Effluent
(on Acidified YMA Medium at 20°C)

Date	Avg. Wastewater Flow (mgd)	Yeast Count per mℓ								
		Filtered and Shaken			Shaken			Blended		
		Incubation, days			Incubation, days			Incubation, days		
		4	6	10	4	6	10	4	6	10
10/05/71	11.9	330	*							
10/12/71	9.5	100	170	200						
10/21/71	8.8	300	500	750						
10/27/71	9.7	95	160	210						
12/07/71	13.1	110	190	270						
12/14/71	14.5	210	250	350	300	600	690	270	550	800
12/16/71	16.4	130	200	200	180	240	380	280	350	360
12/17/71	16.4	90	150	200	250	370	400	260	350	400
Average Value		140	200	280	240	400	490	270	420	520

*Enumeration was not possible because of the spreading of mold growth on the medium surface.

TABLE 8

Total Bacteria and Coliform Content in Raw Municipal Wastewater
(Incubated at 37°C)

Date	Avg. Flow (mgd)	Total Count x 10^6 per mℓ									Coliforms* x 10^6 per mℓ
		Filtered and Shaken			Shaken			Blended			
		Incubation, days			Incubation, days			Incubation, days			
		2	4	6	2	4	6	2	4	6	
9/19/71	10.8	3.00									
9/23/71	10.3	2.30	3.12								
9/30/71	11.6	2.30	2.90								
10/21/71	8.8	1.70	2.30	2.40							
10/27/71	9.7	0.90	1.80	1.90							
12/14/71	14.5	3.70	6.80	7.40	5.20	9.10	10.80	4.30	6.70	7.20	0.33
12/16/71	16.4	1.79	2.60	2.65	5.70	6.10	7.00	4.20	5.00	5.20	0.60
12/17/71	16.4	1.60	2.30	2.40	3.60	4.70	5.10	5.00	6.50	7.75	0.15
Average Value		2.00	2.80	3.00	4.60	6.60	7.60	4.50	6.10	6.70	0.36

*Filtered and shaken samples

TABLE 9

Total Bacteria and Coliform Content in Activated Sludge Effluent
(Incubated at 37°C)

Date	Avg. Flow (mgd)	Total Count x 10^6 per mℓ									Coliforms* x 10^6 per mℓ
		Filtered and Shaken			Shaken			Blended			
		Incubation, days			Incubation, days			Incubation, days			
		2	4	6	2	4	6	2	4	6	
9/19/71	10.8	1.00									
9/23/71	10.3	1.40	2.30								
9/30/71	11.6	1.60	2.20								
10/21/71	8.8	1.80	2.60	2.80							
10/27/71	9.7	1.00	1.50	1.50							
12/14/71	14.5	1.40	2.80	3.10	2.60	3.20	4.40	2.60	4.70	5.30	0.42
12/16/71	16.4	3.20	4.40	4.60	4.00	4.30	4.50	4.20	5.20	5.80	0.96
12/17/71	16.4	2.40	3.20	3.80	2.80	3.70	4.20	2.70	3.30	4.10	0.26
Average Value		1.70	2.60	3.20	3.10	3.70	4.30	3.00	4.40	5.00	0.55

*Filtered and shaken samples

TABLE 10

Total Bacteria and Coliform Content in Trickling Filter Effluent
(Incubated at 37°C)

Date	Avg. Flow (mgd)	Total Count x 10^6 per mℓ									Coliforms* x 10^6 per mℓ
		Filtered and Shaken			Shaken			Blended			
		Incubation, days			Incubation, days			Incubation, days			
		2	4	6	2	4	6	2	4	6	
9/19/71	10.8	1.14									
9/23/71	10.3	1.74	2.72								
9/30/71	11.6	0.62	0.90								
10/21/71	8.8	0.84	1.24	1.50							
10/27/71	9.7	0.85	1.20	1.25							0.17
12/14/71	14.5	0.45	0.80	1.20	1.05	1.30	1.40	2.40	5.30	5.50	0.19
12/16/71	16.4	0.80	1.60	1.85	0.90	1.30	1.42	1.34	2.15	2.50	0.12
12/17/71	16.4	1.50	1.75	1.90	0.73	1.08	1.25	1.90	2.40	2.80	0.14
Average Value		0.95	1.40	1.60	0.89	1.23	1.35	1.85	3.28	3.60	0.15

*Filtered and Shaken Samples

A comparison of the average data of blended samples in Tables 5, 6, and 7 shows a reduction in number of yeast organisms present following secondary treatment, with a greater reduction occurring in the activated sludge process than in the trickling filter treatment. In Tables 8, 9, and 10, the total bacterial count in samples subjected to various pretreatment methods - filtration plus shaking, shaking, and blending alone, is compared. Pretreatment by blending seemed to be the best method of sample pretreatment according to the data presented. The plate counts after blending the sample were higher in general than those after the other two pretreatment methods. It can be seen from Tables 8, 9, and 10 that the coliform content in raw wastewater, activated sludge, and trickling filter effluents ranges from 0.15 to 0.60 x 10^6/mℓ, 0.26 to 0.96 x 10^6/mℓ, and 0.12 to 0.19 x 10^6/mℓ, respectively. It is apparent that the activated sludge process used at the Urbana and Champaign Sanitary District is less efficient in removing coliform organisms than the trickling filter treatment.

A comparison of the limited data available on yeast densities in wastewater as compared to total plate count bacteria and coliforms yields approximate ratios for these organisms. Total plate count bacteria are nearly 4000, 13,500, and 7000 times more numerous than yeasts in raw wastewater, activated sludge, and trickling filter effluent samples, respectively. Coliforms were approximately 200, 1500, and 290 times more prevalent, respectively, in the same samples. Since comparative data for total plate count bacteria, coliforms, and yeasts exist only for filtered and shaken samples which will not reflect organisms associated with large clumps, these ratios should be regarded only as approximations.

Examination of stool samples and wastewater provided about 47 yeast isolates in addition to those obtained from chlorination screening of wastewater. These cultures were randomly selected from assay plates to provide a variety of morphological types and were by no means inclusive of all yeast colonies found on the assay plates. Identification of yeasts is generally based on morphological, physiological, biochemical, cultural and other observations, e.g., shape, size, maximum and minimum, as well as optimum, temperature for budding and sporulation (53). Morphologically these 47 yeast cultures were categorized into four main groups. The first group, and the most frequently encountered, were spherical to oval budding yeasts forming white smooth colonies that exhibit sedimented growth in liquid medium. The second group of yeasts were spherical to oval budding yeasts forming beige colonies and producing a pellicle in liquid medium. The third group of yeasts were elongated to oval budding organisms forming creamy white colonies and producing sedimented growth in liquid media. The last group of yeasts were spherical to oval budding organisms that formed creamy to mucoid pink colonies and yielded sedimented growth in liquid media.

Thirteen of these fecal or wastewater yeast isolates were individually prescreened with 0.5 mg/ℓ free chlorine using chlorine-demand-free water under controlled conditions (Table 11). Four of the cultures tested

TABLE 11

Prescreening for Chlorine Resistance of Yeasts Isolated[a] from Unchlorinated Wastewater Samples or Stools

Culture No.		Time 5 min	15 min	30 min
17 T	(b)	92.5	4.3	0.04
35 T	(b)	0.18	0	0
2 T	(b)	91.7	58.7	0.17
36 T	(b)	45.8	0	0
36 T	(b)	90.5	3.7	4.0
53 RT	(b)	76.8	0.9	0
48	(c)	>90	2.0	0
48	(c)	0	0	0
15	(c)	63.4	11.0	0
10	(c)	6.0	0	0
12	(c)	99.9	25.2	0.16
44 RT	(b)	52.0	0.6	0
52	(c)	2.6	0.1	0

(a) All values were determined at 20°C, pH 7, using 0.5 mg/ℓ free chlorine; results are expressed in percent survival compared to a control

(b) Cultures isolated from wastewaters

(c) Cultures isolated from stools

showed moderate to strong resistance to chlorine. The remainder did not survive 30 min contact time. Representative of each of the four morphological–cultural groups described above were included in these chlorination experiments.

A heterogeneous collection of another 24 yeast isolates was examined in a single mixed culture for chlorine resistance. Nutrient agar slants streaked with pure cultures of yeast were sampled with an inoculating loop and placed in four flasks containing nutrient broth. Yeasts placed in each of the flasks were of a single morphological type based on the morphological classification discussed above. Broth cultures were grown at 25°C for 24 hr on a shaking table. Following growth, the four cultures were sampled volumetrically, the samples combined, and washed several times in sterile phosphate buffer. The washed mixed cells were then used as the inoculum for the chlorination experiment. A free chlorine dosage of 1.05 mg/ℓ was applied. Approximately 1 percent of the total population of yeasts survived after a 5 min contact time. Surviving colonies on YMA were retained for further study. This study indicated that the mixed population of yeasts present in wastewater may be sufficiently resistant to chlorine to provide a basis for a new indicator group for determination of wastewater chlorination efficiency, as opposed to the use of a single species or morphological type.

SELECTIVE TECHNIQUES FOR ENUMERATION OF ACID-FAST BACILLI

Since the acid-fast characteristics of Isolate Nos. 132 and 134 had definitely been established, selective techniques to isolate and to enumerate these two cultures were based principally on methods suitable for *Mycobacterium* isolation. The possibility that these isolates could be members of the genus *Nocardia* was recognized although a limited determinative study indicated that the cultures belonged to the genus *Mycobacterium*.

Isolate No. 132 is a strongly acid-fast bacillus producing yellow, smooth convex subsurface colonies in pour plates. In liquid media, such as nutrient broth, the organism grows homogeneously. The cells appear to be pleomorphic and are arranged singly or in aggregates, large and small. The optimum growth temperature of this isolate was observed to be 37°C, although the isolate was also capable of growing at 10°, 20°, 25°, and 45°C. The isolate was found to be catalase positive but negative for the hydrolysis of casein, gelatin and starch. In addition, the culture utilized acetate, succinate, citrate, malate, oxalate, propionate and pyruvate as a sole carbon source.

Isolate No. 134, the most chlorine resistant isolate, usually grew as thin rods forming small branch-like aggregates, exhibiting a moderate acid-fast characteristic. It produced irregular buff-colored subsurface colonies in pour plates. The optimum growth temperature for this isolate

appeared to be 37°C but growth also occurred at 10°, 20°, 25°, and 45°C. Due to the formation of cellular aggregates, suspensions of this isolate were particulate in nature rather than homogeneous. Addition of Tween 80 to liquid cultures resulted in some degree of dispersal of the cell suspensions. Isolate No. 134 was found to be capable of utilizing acetate, succinate, propionate, citrate, malate, oxalate, and pyruvate as sole carbon sources. Both Isolate Nos. 132 and 134 failed to grow on media containing benzoate and lactate as the sole carbon source. Isolate No. 134 was observed to be catalase positive but negative for casein, gelatin, and starch hydrolysis. Further physiological studies of these two isolates are needed to provide definitive information as to their identity.

A number of studies were carried out in an effort to develop a rapid, sensitive and selective technique for the isolation and enumeration of these acid-fast organisms. These studies included examination of methods of sample pretreatment, study of selective media for acid-fast organisms, and development of a membrane-filter, acid-fast staining technique. The objective was principally to eliminate growth of nonacid-fast organisms in assaying highly contaminated samples such as fecal material and wastewater.

The alkaline sputum treatment procedure generally used as a sample pretreatment method for clinical identification of the tubercle bacilli in sputum specimens (48) was applied to samples of secondary effluents. Samples treated with 1 percent sodium hydroxide showed survival of sporeformers and a few acid-fast bacilli. To eliminate growth of sporeformers on assay plates, the NaOH dosage was increased to 2, 6, and 10 percent. Although growth of sporeformers was suppressed, acid-fast bacilli were also sensitive to this treatment.

Quaternary ammonium compounds (QAC) have been used widely as disinfectants in bacteriology laboratories. Various QAC were, therefore, examined to determine if their use as a sample pretreatment chemical would result in suppressing growth of nonacid-fast contaminants present in wastewater samples. Isolate Nos. 132 and 134 were treated with various QAC at different concentrations and contact times (Table 12). Neither acid-fast isolate survived treatment with Arquad T-2C-50. Both organisms showed some survival to treatment with Armeen C and Isolate No. 134 survived 5 min contact with Armeen 12D. These two QAC, i.e. Armeen C and 12D, were examined further to determine how effectively they suppressed growth of contaminating wastewater organisms. Trickling filter effluent samples were collected, split, and portions treated separately with 0.005 percent of each QAC for 5 min contact. An untreated portion of the effluent was inoculated into Middlebrook and Cohn 7H10 agar pour plates. This untreated sample served as a control. Treatment of effluent organisms with Armeen C resulted in a 50 percent reduction of organisms growing on 7H10 agar as compared to the control. Armeen 12D suppressed growth of 95 percent of the effluent organisms as compared to the controls. A limited number of colonies surviving treatment with

TABLE 12

Effect of Selective Pretreatment Methods on Growth of Acid-Fast Isolates[1]

Pretreatment Chemical and Contact Time	Concentration	Colonies per Plate	% Survival	Colonies per Plate	% Survival
Arquad T-2C-50[2]					
(5 min)	0.005%	0	0	0	0
	Control	190		180	
(10 min)	0.1%	0	0	0	0
	Control	90		250	
Armeen C[2]					
(5 min)	0.005%	54	0	150	87
	Control	190		180	
(10 min)	0.1%	0	0	0	0
	Control	90		250	
Armeen 12D[2]					
(5 min)	0.005%	0	0	120	66
	Control	190		180	
(10 min)	0.1%	0	0	0	0
	Control	90		250	
Oxalic Acid-NaOH					
(5 min)	2.5% Oxalic Acid	47	94	79	30
	Control	50		260	
(5 min)	5.0% Oxalic Acid	15	29	23	9
	Control	50		260	
(30 min)	5.0% Oxalic Acid	1040	59	190	19
	Control	1770		990	

[1] Middlebrook and Cohn 7H10 agar was used as the growth medium in all experiments.

[2] QAC was dissolved in phosphate buffer (pH 7); suspensions were centrifuged immediately after the contact time with QAC and the cells separated from the QAC.

Armeen 12D were randomly selected from the pour plates and were examined for acid-fast properties; 3 of 5 colonies studied were found to be acid-fast positive. Although both Isolate Nos. 132 and 134 showed some resistance to Armeen C, this QAC did not suppress growth of wastewater organisms adequately. While Armeen 12D, on the other hand, suppressed growth of nonacid-fast organisms quite well, Isolate Nos. 132 and 134 were generally sensitive to this QAC. Therefore, the use of QAC as sample pretreatment agents did not appear promising.

However, since it was found that the QAC dissolved more completely in ethanol than in water, further experiments with QAC were carried out using 40 percent ethanol-QAC solutions. Low survival of secondary effluent organisms was obtained when secondary effluent was treated with either 0.005 or 0.05 percent Armeen 12D for 5 min periods. A few colonies examined from the Armeen 12D pretreated assay plate show acid-fastness. Isolate Nos. 132 and 134 were pretreated with ethanol-Armeen 12D solutions and qualitatively appeared to be able to withstand this treatment to some degree.

Use of acid treatment of samples followed by neutralization was studied as a means for reducing the quantity of nonacid-fast organisms on pour plates (Table 12). Incubation of Isolate Nos. 132 and 134 for 30 min in 5 percent oxalic acid, followed by neutralization with 4 percent NaOH, prior to plating in 7H10 agar, gave encouraging results as a sample pretreatment procedure (Table 12). Both treatment contact time and the oxalic acid concentration were varied. Use of 2.5 percent oxalic acid for a contact period of 5 min, followed by neutralization with 2 percent NaOH, resulted in nearly complete recovery of Isolate No. 132. A limited study of Isolate Nos. 132 and 134 seeded in secondary effluent showed that virtually all of the test organisms could be recovered after the oxalic acid-NaOH treatment (2.5 percent oxalic acid for 5 min contact time) while only 0.36 percent of the microorganisms normally present in the effluent survived the same treatment. Therefore, this pretreatment method in isolating acid-fast organisms has been adopted tentatively for analysis of wastewater samples.

Since other microorganisms, especially sporeformers, may survive the oxalic acid-NaOH pretreatment, additional selectivity in the technique is necessary and could be accomplished through the use of a culture medium which is suitable for growth of only the acid-fast organisms present in wastewaters. A search for an appropriate selective medium was undertaken by incorporating various chemotherapeutic agents and dyes into the enriched *Mycobacterium* medium, Middlebrook and Cohn 7H10 agar. Results of survival of Isolate Nos. 132 and 134 on media incorporating penicillin, Eosin-Y, and malachite green are presented in Table 13. Both cultures showed good recoveries when plated in a medium containing Eosin-Y. However, when a sample of a secondary effluent was inoculated on Eosin-Y medium, virtually the same survival of effluent organisms was seen as in control plates containing no Eosin-Y. Since Isolate No. 132 failed to grow in a medium incorporating malachite green, no further study of its

TABLE 13

Effect of Selective Media on Growth of Acid-Fast Isolates[1]

Selective Agent in Media	Concentration	Isolate No. 132 Colonies per Plate	Isolate No. 132 % Survival	Isolate No. 134 Colonies per Plate	Isolate No. 134 % Survival
Penicillin	500 units/mℓ agar	0	0	4	0.4
	1000 units/mℓ agar	0	0	0.5	0.1
	Control	1200		1220	
Eosin-Y	0.005% in agar	380	100	130	76
	Control	310		170	
Malachite green	0.005% in agar	0	0	740	93
	0.0075% in agar	0	0	630	80
	Control	280		800	
Penicillin	10 units/mℓ agar	1	<0.7	650	93
	Control	150		700	
Eosin-Y	0.005% in agar	150	100	710	100
	Control	150		700	
Penicillin Eosin-Y	(10 units/mℓ agar) (0.005% in agar)	0	0	710	100
	Control	150		700	

[1]Selective agents were incorporated into Middlebrook and Cohn 7H10 agar at concentrations indicated.

selectivity was carried out.

Penicillin treatment at concentrations of 25, 50, 100, and 250 units/mℓ of 7H10 agar, as well as a combination of heat and penicillin treatment, all resulted in the growth of many sporeformers. Isolate Nos. 132 and 134 apparently could not survive the concentrations of penicillin used. Thus, it appears that the use of penicillin is not feasible as a selective agent.

Evaluation of 8 antibiotics with sensitivity discs indicated that, as a whole, antibiotics were not suitable since most inhibited Isolate Nos. 132 and 134. The sulfa drugs, on the other hand, showed some promise since both isolates were observed to tolerate the drug concentrations impregnated on the discs. Solutions of 5 different sulfa drugs were therefore incorporated separately into Middlebrook and Cohn 7H10 agar and tested against the acid-fast cultures as well as activated sludge effluent. Results obtained are presented in Table 14. Isolate No. 132 was generally sensitive to all the sulfa drugs tested. Since the sulfa drugs did not dissolve completely in the phosphate buffer used, the actual concentration of the drugs in the agar was not precisely known. The concentration of sulfa drugs in the vicinity of the sensitivity discs on agar plates depends on the rate of diffusion of the sulfa drugs into the media and was, therefore, not precisely known. Thus, the results using the sensitivity discs, and the studies using sulfa drug suspensions should not be compared. In summary, the use of these chemotherapeutic agents did not appear feasible since the actual concentration of drugs could not be controlled accurately.

Since the carbon utilization studies indicated that the acid-fast isolates under study were able to grow on simple mineral media with a simple carbon source, it was felt that this knowledge might be applied to the development of a selective culture medium for these organisms. Since Isolate Nos. 132 and 134 grew on mineral salt medium containing paraffin as the sole carbon source, this medium was tested for its selectivity for acid-fast bacilli in wastewater samples. Difficulty was encountered in dispersing the medium in pour plates although its selectivity appeared to be satisfactory. Acetate and propionate were also selected for use in single carbon source media. These organic compounds were incorporated into Koser's citrate medium as a replacement for citrate as the carbon source. Selectivity for acid-fast bacilli was demonstrated in that growth of sporeformers was suppressed. Results using raw sewage and acetate media showed a variety of morphological and colonial forms. Those cultured on propionate appeared to be morphologically similar to acid-fast Isolate Nos. 132 and 134. Moreover, the selective medium using propionate showed greater inhibition of sporeformers and other nonacid-fast wastewater organisms than the acetate medium. Colonies were randomly picked from propionate medium plates inoculated with raw wastewater and streaked individually on Middlebrook and Cohn 7H10 agar medium. Pure cultures produced on this medium were sampled and tested for acid-fast

TABLE 14

Effect of Sulfa Drugs in Selective Media on Growth of Acid-Fast Isolates

Chemotherapeutic Agents	Apparent Conc. of Drug in Water (mg/ℓ)	Isolate No. 132		Isolate No. 134		Microorganisms in Activated Sludge Effluent	
		Counts/ Plate	Survival %	Counts/ Plate	Survival %	Counts/ Plate	Survival %
No drug present	Control	72		290		310;280	
Kynex	1	22	30.6	330	100	280	93
	5	0	0	310	100	160	52
	10	0	0	330	100	140	47
Sulfathiazole	1	0	0	39	13.5	200	66
	5	0	0	1	0.3	120	40
	10	0	0	0	0	73	25
Gantrisin	1	0	0	310	100	250	84
	5	0	0	290	100	150	51
	10	0	0	290	100	160	55
Sulfamethiozole	1	0	0	320	100	250	85
	5	0	0	280	89.8	130	44
	10	0	0	13	4.1	93	31
Elkosin	1	54	75	310	100	230	76
	5	0	0	310	100	210	71
	10	0	0	310	100	160	54

organisms. Virtually all colonies found on propionate-mineral medium were acid-fast.

The possibility of using the membrane filtration technique after a selective pretreatment to determine the presence of acid-fast bacilli was investigated. Determination of acid-fast bacilli was done by means of a modified acid-fast staining procedure. The result is applicable here because only acid-fast organisms can retain the primary stain after the acid-alcohol decolorization treatment. Samples were pretreated with oxalic acid, incubated, and neutralized as described above.

While the resulting data were limited, selective culturing of test samples on membrane filters using a propionate-mineral growth medium following oxalic acid-NaOH pretreatment provided the potential selectivity and sensitivity necessary to allow the use of acid-fast organisms as a possible bioindicator on a routine basis. The examination of several different raw sewage samples indicated that there were significant quantities of acid-fast bacilli present. Counts of 137 to approximately 500 acid-fast bacilli/mℓ of raw wastewater were observed using this procedure. Limited studies of wastewater effluent samples did not provide reliable results due to considerable quantities of finely divided suspended matter present on the filters. Several types of colonies were present including beige, dry-rough colonies, two types of creamy smooth colonies, and yellow smooth colonies. Creamy and yellow colonies were found to consist of organisms which were strongly acid-fast. While the beige type colonies indicated organisms which were definitely acid-fast, they showed a weaker reaction. The yellow colonial type appears to be similar to Isolate No. 132. The beige variety in pure culture showed a significant percentage of survival after 30 min contact with 1 mg/ℓ free chlorine. This variety showed certain resemblances to Isolate No. 134. The interference by suspended matter in enumerating effluent samples may have been due to insufficient dilution of samples since the suspended matter seemed to show a limited degree of positive acid-fast staining. The literature indicates that *Mycobacterium* is not inactivated during conventional wastewater treatment (22, 24, 37) and therefore should be present in the effluent in concentrations approximating those found in raw sewage. The dilutions used in effluent studies were 100 fold lower than those employed for raw sewage. As a result the few assay plates obtained were probably overgrown with acid-fast organisms.

The combination of a selective pretreatment and a selective culturing method in the order of oxalic acid-NaOH pretreatment, membrane filtration, incubation of the filter on propionate-mineral medium and acid-fast staining of the filter appears to be promising for the isolation and enumeration of acid-fast organisms from wastewater, but further study is necessary to refine the methods and to more fully evaluate its accuracy.

SECTION VI

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